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New approach to *in vitro* culture of animal cells and Tissue engineering

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To Mahmuda sultana - my wife and to Sabyasachi Siddiq - my only son.

This work would never be possible without their patience and constant support.

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Abstract

Objective: The overall theme of this thesis is to investigate the possibility of constructing a complete artificial system to culture cells at the liquid│liquid interface based on the same natural principle of embryogenesis as observed in avian eggs. From a technical and biotechnological perspective, the chicken can be seen as an interface of two immiscible liquids, where the blastoderm develops at the interface between a protein rich in water (egg white / albumen) and lipid (egg yolk). Adopting this natural principle in the laboratory could remove the drawbacks and revolutionize the field of traditional cell culture methods as well as tissue engineering. Unlike mammals the chicken embryo is complete in terms of the nutritional requirements for the developing embryo and independent of the mother animal. This was the reason behind the selection of chicken egg as a model system to study the liquid│liquid interface. The natural egg-system is reconstructed as a bioreactor for culturing mammalian cells, adopting the principle that nature uses during embryogenesis for millions of years.

Background: Advancement of *in vitro* cell and tissue culture techniques including isolation of embryonic stem cells, discovery of adult stem cells and their multi-lineage differentiation raise new hopes in the field of medicine for using these cells in regenerative and transplantation therapy. However, even using the state of the art techniques, it is not possible yet to culture a piece of tissue *in vitro*. The current technology of *in vitro* culture of cells in flasks and on dishes had actually developed from Petri dishes and nutrient-gel-surface culture of microbiology. In such conventional static flat culture flasks or dishes, the two dimensional monolayer environment and plastic substrate tend to alter gene expression and differentiation processes. Cell growth is governed there mainly by the geometry and surface property of the solid substrate. So far the *in vitro* tool for exact cell differentiation comparable to embryogenesis is lacking. The blastoderm swims at a transition zone between two fluids. At this liquid│liquid interface follows the cell division, cellular migration, cell differentiation, and tissue formation dominated by cell microenvironment and orderly cell migration in groups during the process of embryogenesis. Therefore, there should be some potential to copy that principle for *in vitro* cell culture.

Methods: This thesis has a broader aim to construct a complete artificial system for *in vitro* culture of cells at the liquid | liquid interface like in the developing avian eggs. Even though it was not possible to accomplish the mission in the time span of this thesis work, the preliminary investigations were performed in this period, to initiate the work in this field. Emphasis was given to realize the principle of the embryonic growth at the liquid | liquid interface- between egg white and egg yolk of avian egg.

Results: Following the non-invasive study of avian embryogenesis in its natural environment *in ovo* with µMRI, avian embryos were successfully cultured in open culture system consisting of trans-species surrogate shells and were brought to hatching. Modification of the open system allowed complete observation of the development of a chicken embryo from the first day of incubation until hatching. Gradual windowing of the surrogate shell on the side with different biocompatible, optically transparent material revealed the influence of different material properties on the growth of the Chorio-Alantoic Membrane (CAM), which is crucial for embryonic growth and development.

Conclusion: This thesis is the first step towards the overall aim t develop an artificial egg as *in vitro* cell culture system and gives a highly important insight into its feasibility. The results of this thesis indicate that it is possible to construct such a system since it was possible to culture the avian embryos in the open system consisting of surrogate shell from different species. Although many basic problems could be solved, there are still obstacles left that have to be found. These results demand further investigations in this field to fulfill the overall goal of this thesis.

Zusammenfassung

Ziel: Das zentrale Thema dieser Arbeit ist die Suche nach einer Möglichkeit für den Bau eines künstlichen Systems zur Kultur von Zellen in der flüssig/flüssig-Grenzfläche, wie man es in Hühnereiern beobachtet. Das System soll auf dem gleichen natürlichen Prinzip wie die Embryogenese beruhen. Aus technischer und biotechnologischer Sicht entwickelt sich des Hühnerembryo an der Schnittstelle von zwei nicht mischbaren Flüssigkeiten, wobei sich das Blastoderm an der Grenzfläche zwischen einem Proteinbereich in Wasser (Eiweiss / Eiklar) und einem Lipid (Eigelb) entwickelt. Die Anwendung dieses natürlichen Prinzips im Labor konnte bisherige Hindernisse beseitigen und sowohl die traditionellen Zellkulturmethoden sowie das „Tissue Engineerings“ revolutionieren. Was die ernährungsphysiologischen Anforderungen für den sich entwickelnden Embryo und die Unabhängigkeit vom Muttertier angeht, ist der Hühnerembryo -anders als bei Säugern- als abgeschlossenes System zu betrachten. Dies war der Grund für die Auswahl von Hühnereiern als Modellsystem zur Untersuchung der flüssig|flüssig-Schnittstelle. Das natürliche System „Ei“ wird nachgebaut, um als Bioreaktor zur Kultivierung von Säugertierzellen zu dienen. Man bedient sich hierbei des gleichen Prinzips, welches sich seit Millionen von Jahren in der Natur bei der Embryogenese vollzieht.

Hintergrund: Die Weiterentwicklung von *in vitro*-Zell- und Gewebekultur-Techniken, einschließlich der Isolierung embryonaler Stammzellen, der Identifizierung adulter Stammzellen und ihre Differenzierung bezüglich ihrer Abstammungslinien wecken neue Hoffnungen im Bereich der Medizin, wo diese Zellen in der regenerativen Therapie und Transplantation verwendet werden sollen. Aber auch bei dem aktuellen Stand der Technik ist es bis heute nicht möglich, komplexere Gewebebereiche *in vitro* zu kultivieren. Die derzeitige Technologie der *in vitro*-Kultivierung von Zellen in Flaschen und auf Kulturschalen hat sich aus den Petrischalen und Ansätzen der Nährstoff-Gel-Oberflächenkulturen der Mikrobiologiey entwickelt. In solchen herkömmlichen statischen Kulturflaschen oder Kulturschalen neigen die zweidimensionalen Monolayer und die Oberflächeneigenschaften des festen Substrats dazu, die Genexpression und Differenzierungs-prozesse zu beeinflussen. Das stochastische Zellwachstum wird dort hauptsächlich durch die Geometrie und Oberflächenbeschaffenheit der festen Substrate geregelt. Bis jetzt gibt es noch kein *in vitro*-Tool für eine exakte, mit der natürlichen

Embryogenese vergleichbare Zelldifferenzierung. Das Blastoderm schwimmt in einer Übergangszone zwischen zwei Flüssigkeiten. In dieser flüssig|flüssig-Grenzfläche erfolgen Zellteilung, Zellmigration, Zelldifferenzierung und Gewebebildung, welche von der nächsten Umgebung der Zellen und der normalen Wanderung von Zellgruppen während der Embryogenese dominiert werden.

Methoden: Diese Arbeit hat zum Ziel, ein vollständig künstliches System für die *in vitro*-Kultivierung von Zellen in der flüssig|flüssig Grenzfläche wie bei der Entwicklung von Hühnereiern zu schaffen. Obwohl es nicht ganz gelang, dieses Ziel im Zeitrahmen dieser Arbeit zu erreichen, konnten die vorbereitenden Untersuchungen, die für die Arbeit in diesem Bereich notwendig sind, durchgeführt werden. Der Schwerpunkt lag dabei auf der Prüfung aller möglicher Varianten und ersten technischen Realisierungen, die die Tragfähigkeit des Ansatzes belegen.

Ergebnisse: Nach einer nicht-invasiven Untersuchung der Vogel-Embryogenese in ihrer natürlichen Umgebung (*in ovo*) mittels µMRI, wurden Vogelembryonen erfolgreich in einem offenen (avian) Kultursystem bestehend aus künstlichen, speziesunabhängigen Ersatzschalen kultiviert und zum Schlüpfen gebracht. Modifikationen des offenen Systems erlaubten die vollständige Beobachtung der Entwicklung eines Hühnerembryos vom ersten Tag der Inkubation bis zum Schlüpfen. Sukzessive seitliche Fensterung des Schalenersatzes mit verschiedenen biokompatiblen, optisch transparenten Materialien ließen den Einfluss der unterschiedlichen Materialeigenschaften auf das Wachstum der Chorio-alantoic Membran (CAM), die für Wachstum und Entwicklung des Embryos entscheidend ist, erkennen.

Schlussfolgerung: Diese Arbeit beschreibt erfolgreich alle vorbereitenden Versuche bezüglich des übergeordneten Ziels und gibt einen sehr wichtigen Einblick in die Machbarkeit. Die Ergebnisse zeigen, dass es möglich ist, ein solches System bauen, da es möglich war, aviäre Embryonen im offenen System, bestehend aus Schalenersatzmaterial für sogar verschiedener Spezies, zu kultivieren. Obwohl viele grundlegende Probleme gelöst werden konnten, gibt es weiterhin offene Fragen, die erforscht werden müssen. Die Ergebnisse erfordern weitere Untersuchungen in diesem Bereich, um das übergeordnete Ziel dieser Arbeit erreichen zu können und eine praktisch nutzbare umzusetzen.

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List of abbreviations

Abbr.	Extanded abbreviation
µMRI	Magnetic resonance imaging microscopy
3D	Three-Dimensional
ACG	Acoustocardiography
AER	Apical Ectodermal Ridge
ATS	Advanced Tissue Science
BCG	Ballistocardiography
CAM	Chorio Alantoic Membrane
CLSM	Confocal Laser Scanning Microscopy
c-src	cellular onocgen
CSF	Cerebrospinal Fluid
CT	Computed tomography
ECG	Electrocardiogram
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EP	External Pipping
ES	Survival of embryo
FID	Free Induction Decay
FOV	Field of View
HAT	Hypoxanthine Aminopterin Thymidine medium
HSC	Hematopoietic Stem Cell
ICG	impedance-cardiography
ID	Incubation Day
IP	Internal Pipping
ISM	Inner Shell Membrane
ITO	Indium-Tin-Oxide
MAS	Magic Angle Spinning
MRI	Magnetic Resonance imaging
NGF	Nerve Growth Factor
NMR	Nucleaer Magnetic Resonance
NSF	National Science Foundation
OSM	Outer Shell Membrane
P/S	Penicillin/Streptomycin
PCA	poly(e-caprolactone)
PCL	polycaprolactone
PET	Positron emission tomography
PGA	poly(glycolic acid)
PIPAAm	poly(*N*-isopropylacrylamide)
PLLA	polylactic acid
Pt	Platinum
rDNA	recombinant DNA
RH	Relative Humidity
SEM	Scanning Electron Microscopy
SC	stem Cell
SI	shell index
SM	Shell Membrane
SNR	Signal-to-Noise Ratio
SPION	Super Paramagnetic Iron Oxide Nanoparticle
SR	Surface Ratio
T_1	Spin-lattice relaxation time / Longitudinal relaxation time
T_2	Spin-spin relaxation time / Transverse relaxation time
TCPD	Tissue culture polystyrene dishes
TE	Echo Time
ZPA	Zone of Polarizing Activity

1 Introduction

Tissue losses because of injury or diseases and end-stage organ failure are devastating and costly problems in medicine. In ageing population, they reduce quality of life for many at significant socioeconomic cost. All procedures that restore missing tissue in patients require some type of replacement structure for the area of defect or injury. These have traditionally been complete artificial substitutes (joints), non-viable processed tissue (heart valves), or tissue taken from another site from the patients themselves or from other patients (transplantation). Advancement of *in vitro* cell and tissue culture techniques, including isolation of embryonic stem cells, discovery of adult stem cells and their multi-lineage differentiation raise new hopes in the field of medicine to use these cells in regenerative and transplantation therapy. Even using the most advanced techniques, it is not possible yet to culture a piece of tissue *in vitro*. The field of tissue engineering emerged in response to the growing need for tissues and organs for transplantation. Tissue engineering and selective cell transplantation were born as means to replace diseased tissue with a viable one that is "**designed and constructed to meet the needs of each individual patient**". Tissue engineering is no longer restricted to the academic laboratory. Tissue-engineered skin is commercially available; cartilage is in clinical trials and should be available within a few years.

With the advancement of modern science and technology, many new products are developed every day, intended for human use. Product testing and evaluation is an immense issue. For years, the development of appropriate animal and tissue model for testing and evaluation of pharmaceutical and cosmetic products, biomaterials, tissue engineering and for clinical application in regenerative medicine and transplantation therapy has promoted immense programs worldwide. No doubt, *in vitro* cell and tissue culture play a key role in these areas and will find a wider application in animal and human cell models in the field of medicine and biotechnology. The production of vaccines, enzymes, hormones and immunobiologicals revolutionised medicine. Mass cultures of animal cell lines are fundamental to the manufacture of viral vaccines and many products of biotechnology. Biological products produced by recombinant DNA (rDNA) technology in animal cell cultures include enzymes, synthetic hormones,

immunobiologicals (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells.

The current technology of *in vitro* culture of cells in flasks and on dishes has actually developed from the Petri dishes and nutrient-gel-surface culture of microbiology. It is difficult to automate and allows insufficiently defined microenvironment for cells. It is limited and dominated by the technical limitations of the culture dish. In general, these methods are used to culture cells outside the organism. The cells are isolated from tissue/subculture with the treatment of proteolytic enzymes (e.g. Trypsin) and cultured using solid cell-culture substrates in a single cell layer (two-dimensional monolayer culture) at the interface between solid and liquid or in the suspension. This treatment with proteolytic enzyme however may have detrimental effects on cells. Unlike *in vivo*, cells there act as independent units, much like a microorganism such as a bacterium or fungus. The cell growth is governed mainly by the geometry and surface property of the solid substrate and stochastic cell interactions. Instead of forming a three-dimensional tissue like structure *in vivo /in ovo* (as observed in embryogenesis where a single cell is differentiated into three different germ layers and eventually form a complete organism) they proliferate in a monolayer until a single layer of cells just touching each other covers the surface of the culture dish. By contrast in mammalian tissues, cells connect not only each other, but also a support structure called the extracellular matrix (ECM). The growth of normal cells as two-dimensional monolayer on artificial support leads to partial loss of original cell characteristics with the quality of the monolayer being strongly influenced by physiochemical properties of the support. From different experiments it is evident that cell phenotype depends much on its micro-environment. Inappropriate alterations of cell–microenvironment interactions can result in abnormal cellular behaviour. It is quiet evident that the phenotypes of the *in vitro* cultured cells are different than in the physiological state. For these reasons, researchers are now leaning towards culturing cells in three-dimensional environment mimicking the physiological state.

During embryogenesis, single fertilized oocytes gives rise to a multicellular organism whose cells and tissues have adopted differentiated characteristics or fates to

perform the specified functions of each organ of the body. As embryos develop, cells that have acquired their particular fate proliferate, enabling tissues and organs to grow. Even after an animal is fully grown, however, many tissues and organs maintain a process known as homeostasis. As cells die, either by natural death or by injury, they are replenished. This remarkable feature has ancient origins, dating back to the most primitive animals, such as sponges and hydrozoans. Throughout evolution, nature has exerted considerable fun and fancy in elaborating on this theme. Some amphibians, for instance, can regenerate a limb or tail when severed, and the neurons of bird brains can readily regenerate. While mammals seem to have lost at least some of this wonderful plasticity, their liver can partially regenerates providing that the injury is not too severe, and the epidermis and hair of their skin can readily repair when wounded or cut. Additionally, the epidermis, hair, small intestine, and hematopoietic system are all examples of adult tissues that are naturally in a state of dynamic flux: even in the absence of injury, these structures continually give rise to new cells, able to transiently divide, terminally differentiate and die. The fabulous ability of an embryo to diversify and of certain adult tissues to regenerate throughout life is a direct result of stem cells, nature's gift to multicellular organisms.

Stem cell differentiation *in vitro* is, more or less, a stochastic process. Embryonic stem cell lines have the potential to form derivatives of all three embryonic germ layers. *In vitro*, however these cells differentiate when cultured in the absence of embryonic fibroblast feeder layers. When grown to confluence and allowed to pile up in a culture dish, embryonic stem cell lines differentiate spontaneously even in the presence of feeder cells. Controlled differentiation and tissue formation is needed for tissue engineering. This needs defined microenvironments and proper migration of cells. In tissue engineering process, *in vitro* differentiated cells are seeded into a porous bio-degradable scaffold and cultured in a bioreactor to form tissue which is later intended to be implanted into the patient. The use of bio-degradable scaffold material (mimicking the extra-cellular matrix) to construct tissue like structures actually emerged due to the inability to grow tissue from cells *in vitro*. A tissue is composed of different type of cells performing specific functions in co-ordination with each other. Co-culture of different cells also failed to form tissue *in vitro*. Theoretically, the biodegradable scaffold should be reabsorbed and replaced by the proliferated cells and extracellular matrix to form a

complete tissue. However, in reality the situation is quite different. Mass transfer (nutrient and O_2) is the main obstacle in this regard. Since diffusion is the only means of transportation, cells do not penetrate deep into the scaffold and grow only at the interface between scaffold and culture media due to the deficiency of nutrient and O_2. Implantation of such a construct into the patient will not form tissue. Rather scaffold degradation products trigger immunogenic reactions; eventually leading to scar formation.

Currently the tissue engineering approach, particularly the scaffold lacks the physiological boundary conditions and the cellular microenvironment. Actually, the basic principle is similar to *in vitro* cell culture on solid surface. As worth solid | liquid interface culture, there is not enough flexibility for orderly cellular migration. Even with big success in cell culture and increasing understanding of molecular and genetic processes of stem cell differentiation, the results of tissue engineering are lagging far behind of expectations and are not yet satisfactory. So far, the *in vitro* tool for exact cell differentiation comparable to embryogenesis is lacking. Apart from some simple epithelial and endothelial systems- functional, complex and multilayered physiological tissue models are absent from stem cell research. In many cases, the appropriate biological, physical and chemical cues are not yet completely understood.

Developmental biologists knew the complex process of embryogenesis for a long time. The complexity and orderly fashion of tissue formation and organogenesis fascinated them. Throughout history, it attracted great naturalists, artists, philosophers, pioneers of biology and stimulated them to think about the most fundamental questions on generation and life. In ancient times, Aristotle was fascinated by the uniqueness of the chicken egg. In the 19th century, egg from sea urchins, Amphibians, reptiles, fish, and in particular birds eggs dominated in the field of embryonic research. By the end of the 19th century, Wilhelm Roux and his followers realized that carefully designed experimental manipulations that disturb development could provide information about the developmental potential of cells in the embryo. These studies led to the clear notion that development depends upon the flow of signals between different cell populations. In the twenties of the last century, Spemann and Mangold [309] with their ground-breaking experiments on embryos demonstrated that special cell groups take part in

tissue formation and organogenesis. Embryogenesis is the best and perfect example of tissue engineering by the nature.

The blastoderm swims at a transition zone between two liquids. At this liquid | liquid-interface follows the cell division, cellular migration, cell differentiation, and tissue formation during the process of development. During embryogenesis– dominated by cell microenvironment and orderly cell migration in groups – a single fertilized oocytes gives rise to a multicellular organism whose cells and tissues have adopted differentiated characteristics or fates to perform the specified functions of each organ of the body. As the embryo develops, cells that have acquired their particular fate proliferate, enabling tissues and organs to grow. Apparently, this three-dimensional freedom of movement facilitates cell division and migration as well as far-reaching freedom for the developing embryo. This remarkable feature has an ancient origin and has attained perfection throughout million of years of evolution. Nature has expended considerable fun and fancy in elaborating on this theme. Until now, not much attention has been paid to this issue. Cell culture methods and stem cell research has rather deviated from the nature and is based on artificial methods.

On one hand, the embryologists continues to watch the fascinating process of development. On the other *in vitro* cell culture and stem cell research continues on an artificial non-physiological platform. Different scientists have mentioned this historical gap between cell research and developmental biology for a long time. It therefore appears as almost mandatory to bridge the gap, to seek for alternatives to the *in vitro* cell culture approache that avoid the disadvantages of conventional solid surface cell culture systems and develops a more flexible and physiological cell culture system whose boundary conditions are controllable.

1.1 *In vitro* cell culture

As a routine procedure in the laboratory, animal cells are removed from tissues or previous cultures with enzymatic digestion and placed on solid cell culture dishes (treated or non-treated) covered with culture medium containing appropriate nutrients. In appropriate culture conditions, cells grow and become confluent covering the whole solid surface of the culture dish. Cell growth and phenotype is often governed by the

physiochemical boundary conditions of the culture environment. As opposed to the *in vivo* situation (forming three dimensional tissue), *in vitro* culture cells grow in a mono layer covering the whole surface of the culture dish just touching each other (other then suspension culture or hanging drop preparations). The culture process allows single cells to act as independent units, much like microorganisms such as a bacteria or fungus.

Cells are capable of dividing; they increase in size and, in a batch culture, can continue to grow until limited by some culture variable such as nutrient depletion. When normal diploid fibroblasts growing on a glass surface come into contact, an adhesion forms and cell movement in that direction stops. As the resulting "monolayer" of diploid cells becomes confluent, their growth rate also decreases markedly [187]. The conventional process of cell culture is actually adapted from the microbial culture process of the microbiology.

Robert Koch (1843–1910) developed the method for isolation of bacteria in pure culture that consisted essentially of semisolid medium, a nutrient environment solidified by the addition of gelatine or agar-agar, a method so simple and yet so effective that is used practically unchanged today. By 1887, Julius Richard Petri, one of Robert Koch's assistants, introduced the Petri dish. This simple invention provided a far more versatile means of culturing microorganisms than did use of the bulky bell jars employed previously. Louis Pasteur (1822–1895) introduced the first semi-synthetic medium designed for cultivating bacteria in 1860 by which replaced the previous use of meat broths as bacterial growth medium, an approach that persisted well into this century. From 1898 onward, the art of enrichment culture was developed. This led to the isolation of both nitrifying and cellulolytic bacteria. The process of cell or tissue culture has been adopted from all these methods of microbiology.

The field of cell and tissue culture has gradually developed over last century, and continues to make rapid strides because of improvement in techniques, and application of the experimental results of Biochemistry and Microbiology. The work of Arnold [382] proved that animal cells could survive for a short time outside the animal body. Ross Harrison [117-120] explanted fragments of tadpole spinal cord in Lymph thereby demonstrating that axons are produced as extensions of single nerve cells. Gradually

different types of culture flasks and bottles were developed. Different plastic and synthetic materials are also employed for manufacturing culture ware.

Table 1-1: Some Landmarks in the Development of Tissue and Cell Culture

1885	Roux shows that embryonic chick cells can be maintained alive in a saline solution outside the animal body.
1907	Harrison cultivates amphibian spinal cord in a lymph clot, thereby demonstrating that axons are produced as extensions of single nerve cells.
1910	Rous induces a tumour by using a filtered extract of chicken tumour cells, later shown to contain an RNA virus (Rous sarcoma virus).
1913	Carrel shows that cells can grow for long periods in culture provided they are fed regularly under aseptic conditions.
1948	Earle and colleagues isolate single cells of the L cell line and show that they form clones of cells in tissue culture.
1952	Gey and colleagues establish a continuous line of cells derived from a human cervical carcinoma, which later become the well-known HeLa cell line.
1954	Levi-Montalcini and associates show that nerve growth factor (NGF) stimulates the growth of axons in tissue culture.
1955	Eagle makes the first systematic investigation of the essential nutritional requirements of cells in culture and finds that animal cells can propagate in a defined mixture of small molecules supplemented with a small proportion of serum proteins.
1956	Puck and associates select mutants with altered growth requirements from cultures of HeLa cells.
1958	Temin and Rubin develop a quantitative assay for the infection of chick cells in culture by purified Rous sarcoma virus. In the following decade, the characteristics of this and other types of viral transformation are established by Stoker, Dulbecco, Green, and other virologists.
1961	Hayflick and Moorhead show that human fibroblasts die after a finite number of divisions in culture.
1964	Littlefield introduces HAT medium for the selective growth of somatic cell hybrids. Together with the technique of cell fusion, this makes somatic-cell genetics accessible.
	Kato and Takeuchi obtain a complete carrot plant from a single carrot root cell in tissue culture.
1965	Ham introduces a defined, serum-free medium able to support the clonal growth of certain mammalian cells.
	Harris and Watkins produce the first heterocaryons of mammalian cells by the virus-induced fusion of human and mouse cells.
1968	Augusti-Tocco and Sato adapt a mouse nerve cell tumour (Neuroblastoma) to tissue culture and isolate clones that are electrically excitable and that extend nerve processes. A number of other differentiated cell lines are isolated at about this time, including skeletal muscle and liver cell lines.
1975	Köhler and Milstein produce the first monoclonal antibody-secreting hybridoma cell lines.
1976	Sato and associates publish the first of a series of papers showing that different cell lines require different mixtures of hormones and growth factors to grow in serum-free medium.
1977	Wigler and Axel and their associates develop an efficient method for introducing single-copy mammalian genes into cultured cells, adapting an earlier method developed by Graham and van der Eb.

Carrel [44-50] showed that cells could grow for long periods in culture provided they are fed regularly under aseptic conditions. In 1928 Maitland [193] first introduced the

method of growing tissue in culture, a method developed mainly for the growth of viruses. White [386-388] in 1946 made one of the first attempts to grow animal tissues in synthetic solution of known composition. This was used for the growth of chick embryo tissue and he managed to get cells to survive for some weeks. This medium included in its composition a very wide range of amino acids and vitamins made up in a balanced salt solution base, and supplemented still further with a number of nucleic acid constituents and other substances thought to be growth promoting. Basically, the idea was not new, actually developed nearly a century ago by Louis Pasteur intended for bacterial culture.

In 1998 Thomson [339], Gearhart [300] and their associates isolated human embryonic stem (ES) cells. Their use in research as well as therapeutics is encumbered by ethical considerations and discovery of adult stem cell raised new hopes for using these cells for medical therapy. They have the potential to differentiate into all cell types. Anticipation was to differentiate stem cells *in vitro* into desired cell types and then to use them for therapy. However, the stem cell differentiation in *in vitro* culture is rather a stochastic process than a controlled differentiation into desired cell type. In *in vitro* culture, embryonic stemcells need embryonic fibroblast feeder cells to keep them in undifferentiated state. Celllines differentiate spontaneously even in the presence of feeder cells. Researchers are facing significant challenges in these efforts because stem cells are difficult to handle and there are very few automated or standardized tools available in this relatively new field.

1.1.1 Ambiguous behaviour of cells in different culture environments

Cellular microenvironment and the boundary conditions play a very important role on cellular phenotype and physiology. Providing an artificial, non-physiological microenvironment may alter the cellular phenotype than *in vivo*. Especially in the field of stem cell research, environment plays a very important role in differentiation process. Inappropriate alterations of cell–microenvironment interactions can result in abnormal cellular behaviour, as seen in tumour progression [283]. For example, *in vitro* embryonic stemcells need embryonic fibroblast feeder cells to keep them undifferentiated. When grown to confluence and allowed to pile up in the culture dish, the embryonic stemcell lines differentiate spontaneously even in the presence of feeder cells. An interesting

example of cell environment affecting differentiation occurs with the ectopic implantation of embryonic stemcells transforms them into malignant tissues while the same cells located in the uterus undergo normal embryogenesis [197].

In conventional static flat culture flasks or dishes, the two dimensional monolayer environment and plastic substrate tend to alter gene expression and prevent differentiation [1, 71]. Most of these gentle cell culture techniques produce flat, one-cell-thick specimens that offer limited insight into how cells work together. In mammalian tissues, cells connect not only to each other but also to a support structure called the extracellular matrix (ECM). This contains proteins, such as collagen, elastin and laminin, which give tissues their mechanical properties and help to organize communication between cells embedded within the matrix. Receptors on the surface of the cells, in particular a family of proteins called the integrins, anchor their bearers to the ECM, and also determine how the cells interpret biochemical cues from their immediate surroundings [1]. Given this complex mechanical and biochemical interplay, it is perhaps no surprise that researchers will miss biological subtleties if the cells they are studying grow only in flat layers. But providing an appropriate environment in which to culture cells in three dimensions is no easy matter. There is a big difference between a flat layer of cells and a complex, three-dimensional tissue.

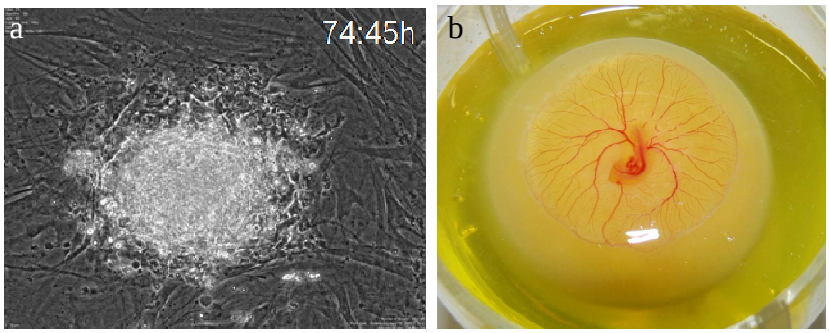

Figure 1.1: Influence of culture environment on cells

Embryonic stem cells after ≈75 hours of incubation: (a) Human embryonic stem cell cultured *in vitro* with fibroblast feeder cells after ≈75 hours of incubation; (b) embryonic stem cell develops to a chicken embryo *in ovo* after same periods of incubation (video supplied). Image (a) adhesion and proliferation of a H1 hESC-colony (H1 line provided by WISC bank, WiCell research institute, USA) on inactivated PMEF for 96 hours. This experiment has been conducted as control for analysing the effects of slow-rate cryopreservation on the adhesion ability of H1 within the EU-project CRYSTAL, FP6-037261 (Robert-Koch permission No. 18). Live-cell imaging was prepared and analysed by F. Groeber and M. Gepp, from "Biophysics & Cryotechnology department" of Fraunhofer IBMT.

Two-dimensional monolayer culture models lack the three-dimensional microenvironment of intact tissue. A major limitation of the two-dimensional monolayer is lack of stroma. Stroma of the mammary gland accounts for more than 80% of the resting breast volume [71]. Monolayer culture also lacks structural architecture and significant limits of transport. Cells cultured using traditional two dimensional monolayer techniques frequently undergo phenotypic and functional de-differentiation [1]. After a period of continuous growth, cell characteristics can change and may become quite different from those found in the starting population. Cells can also adapt to different culture environments (e.g. different nutrients, temperatures, salt concentrations etc.) by varying the activities of their enzymes. It is, possible that the cellular fates generated by adult stem cells are restricted because of the limitations imposed on them by the particular environment in which they have been evaluated.

The necessary components include both regeneration-competent cells and the carrier or support matrix. Another requirement is an environment conducive to cell growth, differentiation and eventually integration with the surrounding tissue. Most, if not all differentiated cells derived from diverse tissue sources lose their specialized features and dedifferentiate when grown under traditional two-dimensional cell culture conditions [12, 375, 383, 390]. Scientists are starting to realize just how much a cell's context matters and are now trying to mimic the three dimensional environment and to grow cells in three-dimentional culture. Cells are embedded in a structure that mimics the extracellular matrix (ECM) of structural proteins and other biological molecules found in real, viable tissues. Many researchers use a material called Matrigel, a cocktail of substances extracted from the ECM of a type of mouse tumour and first described some two decades ago [160, 161].

From different experiments, it is quiet evident that the culture conditions and cellular microenvironment influence cell behaviour and phenotype. Normal epithelial cells when grown in monolayer, are highly plastic and express many characteristics displayed by tumour cells *in vivo* [27, 256]. Growth of normal epithelial cells as two dimensional monolayer on artificial supports leads to partial loss of the original epithelial cell characteristics with the quality of the monolayer being strongly influenced by the physicochemical properties of the support [157]. Moreover, without a proper three-dimensional (3D) assembly, epithelial cells (the basic cells that differentiate tissue into

specific organ functions) lack the proper clues for growing into the variety of cells that make up a particular tissue[177]. Human mammary epithelial cells, isolated from reduction mammoplasty, grown in culture on reconstituted basement membrane form polarized acinus type structures capable of gland specific function such as milk production [144, 317]. However, the same cells grown in a different substrate, type 1 collagen, show altered integrins, abnormal cellular polarity and disorganization emphasising the importance of matching cell type with appropriate substrate [132]. Antibodies against a cell surface receptor called β1-integrin completely changed the behaviour of cancerous breast cells grown in three-dimensional culture; they seemed to become non-cancerous, losing their abnormal shapes and patterns of growth [383]. This result had never been observed in two-dimensional cultures. In the same breast-cancer system, it has been shown that antibodies against β1-integrin also decrease signalling by receptors for epidermal growth factor (EGF); antibodies against EGF receptors similarly depress the activity of β1-integrin [375]. This reciprocal interaction does not happen in two-dimensional cultures. When grown in three-dimensional cultures, human disc cells form multicelled colonies. When grown in monolayer culture, human disc cells assume a flattened, spindle-shaped morphology [108].

The cell-surface receptors to which adenoviruses bind have been investigated. In two- dimensional cultures, both normal and malignant breast cells had similar, high levels of the receptors. But in three-dimensional cultures, only malignant cells carried a large numbers of the receptors [12]. The growth and development of fibroblasts, collagen-secreting cells that are found in many tissues directly compared, in two-dimensional and three-dimensional cultures. In three dimensions, the cells moved and divided more quickly, and assumed the characteristic asymmetric shape that fibroblasts have in viable tissues [63]. Implantation of stem cell from different species into the chicken embryo shows region specific differentiation. When hematopoietic stem cells (HSCs) from adult human bone marrow are implanted into lesions of the developing spinal cord in the chicken embryo, the human cells never express a chicken-specific antigen, but differentiate into full-fledged neurons. The microenvironment of the regenerating spinal cord of the chicken embryo stimulates a substantial proportion of adult human HSCs to differentiate into full-fledged neurons [304].

Two-dimensional monolayer culture models are easy and convenient to set up with good viability of cells in culture. Many of the seminal findings in cell and molecular biology have come from cultures of cells grown cheaply and conveniently in these familiar, surface cultures. However, the limitations of just two dimensions are now becoming clear. Researchers are now in search of alternative system for *in vitro* culture of cells that mimic more physiological conditions.

The fetal brain, characterized by active neurogenesis, has been suggested to be a promising source of therapeutic neural stem cells [90]. Such cells have also been suggested as potential therapies for infants and children affected by genetic and acquired diseases characterized by neurological deterioration [90, 105]. Scientists think, however, that it might be possible to use ‘‘neural stem cell’’ transplants to replace the neural cells that are lost in neurodegenerative diseases (for example, Parkinson’s disease) or damaged by strokes or trauma. The injection of pluripotent ESC or ESC-derived precursor cells in rodents leads frequently to the development of teratomas or teratocarcinomas [28, 84, 272]. Although the tumorigenic potential of ESC seems to be greatly reduced when cells are predifferentiated in vitro before implantation [19, 36, 271]. Importantly, ESCs seem more prone to generate tumors when implanted into the same species from which they were derived [84]. However, a recent study has raised questions regarding the safety of stem cell therapy [10]. An ataxia telangiectasia patient (a rare disorder characterized by degeneration of the brain region that controls movement and speech, occurs when both copies of the ATM gene contain a genetic change that stops the production of functional ATM protein) had repeated transplantation of fetal stem cells into the brain and the CerebroSpinal Fluid (CSF). Later the patient developed tumour. Histo-pathology and immunological examination of the tumor confirmed to be a glioneuronal tumor containing both XX (female) and XY (male) cells and the tumor contained cells from at least two donors. This also raise question about tissue engineering, where the implantation of tissue engineered construct may also lead to tumor formation. Other important issue remains unclear about the *in vitro* differentiated stem cell used in tussue engineering. Since cells are differentiated *in vitro* in a non-physiological environment, cells may dedifferentiate, redifferentiate or even form tumor *in vivo* after implantation into the body.

1.2 Tissue engineering

Over the last 50 years, transplantation of a wide variety of tissues, reconstructive surgical techniques, and replacement with mechanical devices have significantly improved patient outcomes. Murray and colleagues performed the first successful organ transplant in 1954 [359]. Since that historic accomplishment, the field of transplantation has evolved to include kidney, liver, split liver, pancreas, heart, lung, and small intestine at hundreds of transplant centres throughout the world. In 1967, Barnard performed the first heart transplant for congestive heart failure [24]. These strides have been made possible because of the advances in transplantation biology and immunology leading to the development of a variety of immunosuppressive agents. Unfortunately, organ and tissue transplantation are imperfect solutions because they are limited by a number of factors. Worsening donor shortages result in a discrepancy between the number of patients needing transplants and available organs. Today, donated organs and tissues are often used to replace those that are diseased or destroyed. The number of people needing a transplant far exceeds the number of organs available for transplantation. Additionally, transplantation recipients must follow lifelong immunosuppression regimens with their increased risks of infection, tumour development, and unwanted side effects. Surgical reconstruction also suffers from a lack of available donor tissue and donor site morbidity. Replacement with mechanical devices or artificial organs is limited by an increased risk of infection, thromboembolism and finite durability.

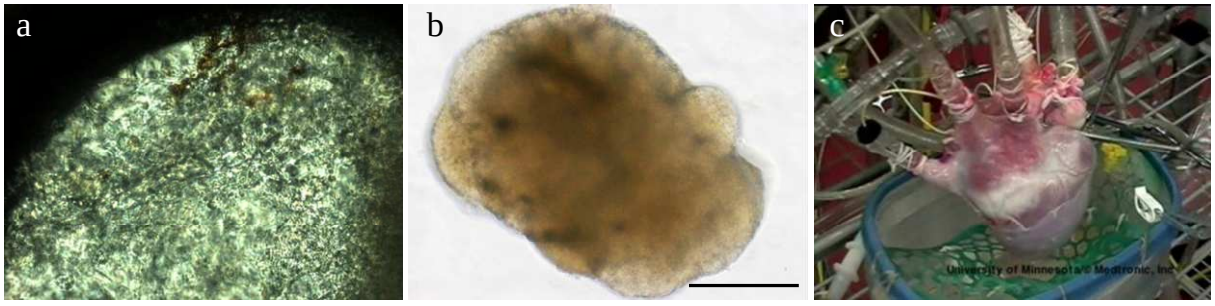

Figure 1.2: Draw back of conventional *in vitro* cell culture and stem cell research.

(a) Cardiomyocyte differentiated from stem cell, (b) cardiac spheroid, (c) porcine heart in the laboratory. It is possible to differentiate stem cell into cardiomyocytes, which can form a beating spheroid. However, forming a complete organ or tissue out of it in the laboratory is not possible until now (video supplied). Scale Bar in image (C) = 75µm. Image (a) courtesy of Dipl.-Biol. Rothin Strehlow from workgroup "Cell Programming and Bioinformatics" of Fraunhofer IBMT in Potsdam-Golm, Germany; Image (b) courtesy of M.Sc. Ina Meiser from "Biophysics & Cryotechnology department" of Fraunhofer IBMT in St. Ingbert; Image (c): courtesy of Prof. Paul A. Iaizzo, PH.D., Visible heart Lab, University of Minnesota.

Advances in stem cell research, especially plasticity of adult stem cell raised new hopes for tissue construction for medical therapies. Adult stem cells, such as blood-forming stem cells in bone marrow (called hematopoietic stem cells, or HSCs), are currently the only type of stem cell commonly used to treat human diseases. Doctors have been transferring HSCs in bone marrow transplants for over 40 years, and advances in techniques of collecting, or "harvesting" HSCs have been made. HSCs are used to reconstitute the immune system after leukaemia, lymphoma or various blood or autoimmune disorders have been treated with chemotherapy. However, even, using the most advanced techniques it is not yet possible to culture a piece of tissue suitable for transplantation using these cells. In traditional culture, cells grow, differentiate but remain as cells. Because of the above shortcomings, the field of tissue engineering and selective cell transplantation was born as a means to replace diseased tissue with viable one that is "designed and constructed to meet the needs of each individual patient". Tissue engineering is "an interdisciplinary field that applies the principles and methods of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [176, 360].

The term "**Tissue Engineering**" was introduced in 1987 by members of the US National Science Foundation (NSF) in Washington, D.C. It was defined a year later at an NSF organized conference on tissue engineering in Lake Tahoe, California as **"Application of principles and methods of engineering and life sciences toward fundamental understanding of structure–function relationship in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions."** Tissue engineering aims at generating functional three-dimensional tissues outside of the body that can be tailored in size, shape and function according to the particular needs before implanting them into the body. Tissue engineering is no longer restricted to the academic laboratory. Tissue-engineered skin is commercially available; cartilage is in clinical trials and should be available within a few years. First clinical experiences have been published using bioengineered skin, cartilage, and vascular grafts [100, 266, 303], but the present data are still preliminary [401].

Three general tissue-engineering approaches have been attempted thus far. These include guided tissue regeneration using engineered matrices alone, the injection of allogenic or xenogenic cells alone, or the use of cells placed on or within matrices [176, 360]. The latter two methods are the most common. The second approach can be synonymous with, "cell therapy" which intends to promote the formation of new tissue or to improve the function of an existing tissue by injecting or infusing suspensions of isolated cells. This concept has gained much attraction over the past years; studies have been performed in animals [53, 164, 167, 168, 307] and are currently tested in controlled clinical trials [70, 173, 207, 208, 220, 221, 318, 370]. This approach, however, may be of little clinical benefit when the local organ structure cannot support cell seeding because it is missing or seriously damaged, it is difficult to control shape, size and location of the grafted cells. Additionally, isolated cell transplantation is not enough for replacing congenital defects [302]. It has the drawbacks of possible rejection or loss of function [176].

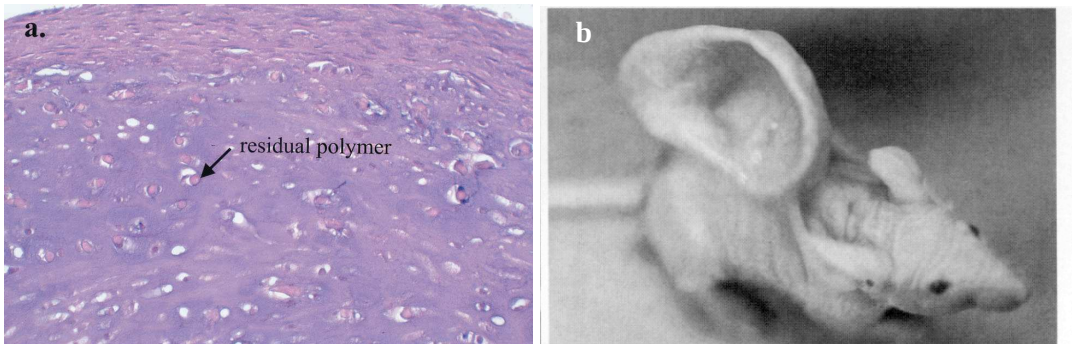

Figure 1.3: Tissue-engineered cartilage

(a) Photomicrograph of tissue-engineered cartilage using fetal chondrocytes from ear specimens. Arrow = residual polymer. (Haematoxylin and eosin) [99]. (b) Tissue engineered cartilage as the shape of human ear 12 weeks after subcutaneous implantation into a nude mouse [47].

The use of cell-matrix constructs, the most common method in tissue engineering, involves either an open or a closed system. An open system begins with the *in vitro* culture of isolated cells. The cells are then seeded onto a scaffold or matrix, either synthetic or natural. After appropriate cultivation time, the cell-matrix construct is implanted into the host. The matrix functions to guide the development of the new tissue and provides structural support. This approach is based on a number of biological observations. Firstly, all tissues undergo constant remodelling. Under appropriate environmental conditions, dissociated cells often reform their native structures [359].

Furthermore, normal parenchymal cells are anchorage dependent; they also require three-dimensional structure and an extracellular matrix to guide their growth. Lastly, the volume of tissue that can be implanted and survive is limited by the diffusion distance for nutritional molecules, gas exchange, and waste removal [359]. In a closed system, the cells are isolated from the body by a permeable membrane allowing exchange of nutrients and waste but protecting the cells from the immune response [176].

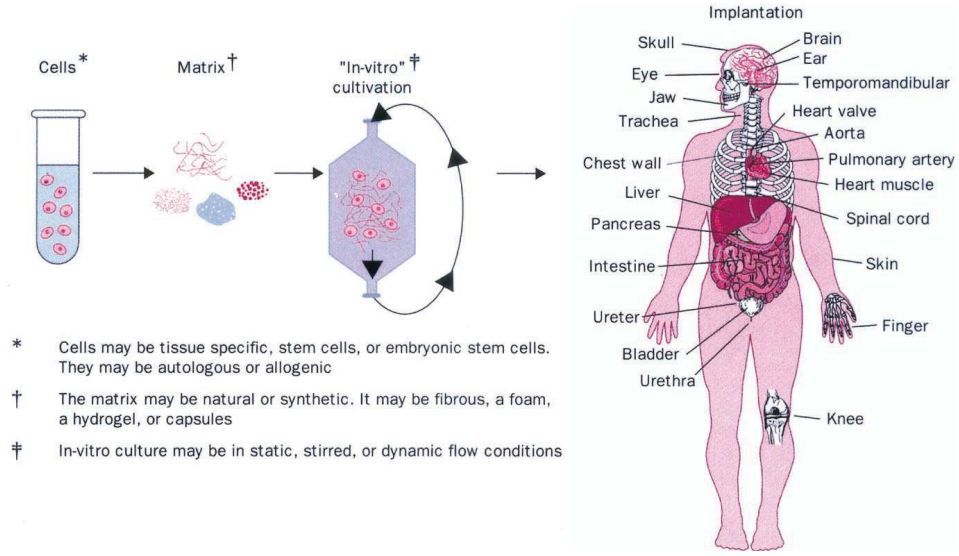

Figure 1.4: The tissue engineering.

Schematic representation of Tissue engineering process[360].

1.2.1 State of the art

Important procedures in tissue engineering include the isolation and selection of organotypic cells from small tissue biopsies, their *ex vivo* proliferation by cell culture techniques in bioreactor, and the seeding of these cells into suitable biocompatible matrices. These constructs are commonly transferred from the *in vitro* situation into an *in vivo* state (transplantation). Tissue engineering has currently been based on the concepts that three-dimensional biodegradable scaffolds are useful as alternatives for extracellular matrix (ECM) and that seeded cells reform their native structure in according to scaffold biodegradation [176]. This context has been used for every type of tissue. The word "biodegradation" is defined to be the phenomenon where a material is degraded or water solubilised by any process in the body to disappear from the site implanted [324].

Synthetic and natural polymers are attractive alternative and versatile in their applications to the growth of most tissues. Among Synthetic polymers, aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLLA), their copolymers (e.g. PLGA) and polycaprolactone (PCL) are most commonly used for tissue engineering scaffold applications [3, 135, 136, 371]. The degradation products of these polymers (glycolic acid and lactic acid) are present in the human body and are removed by natural metabolic pathways. Naturally derived protein or carbohydrates (natural polymers) have been used as scaffolds for the growth of several tissue types. Most natural hydrogels, such as collagen, gelatin, alginate, fibrinogen, chitosan, and carrageenan are compatible with cells.

1.2.2 Limitations of current tissue engineering approach

Engineered skin and cartilage have recently been introduced for clinical use [169, 358]. The manufacture of Dermagraft® by Advanced Tissue Sciences (ATS) was a pioneering example of the use of bioreactors in the large-scale production of tissue-engineered products [134]. Although some structural tissues have been made, such as skin, bone, and cartilage, there are few results that developed complicated bioactive organs, such as kidney or liver. The low oxygen requirement of cartilage may be the reason why only this tissue has been successfully grown *in vitro* to thick cross-sections i.e. greater than 1mm using conventional scaffold fabrication techniques [371]. Even though the largest organ of the body, skin is relatively two dimensional tissue and thus thick cross-sections are not required, thereby explaining the success of producing this tissue with conventional scaffold fabrication techniques [76]. However, most other three dimensional tissues require a high oxygen and nutrient concentration. Long-term survival and function of such three -dimensionally constructed tissues depend on rapid development of new blood vessels, which provide nutrients and oxygen not only to the marginal cells but also of the centre of the tissue grafts. In fact, the growth of a new microvascular system remains one of the major limitations in the successful introduction of tissue engineering products to clinical practice. Accordingly, the focus of research in tissue engineering has changed toward the understanding of angiogenesis and new blood vessel formation. The limiting factor for the survival, proliferation, and differentiation of transplanted cells is sufficient supply of nutrients and oxygen. This supply relies on

diffusion processes. Furthermore, to supply tissue-engineered constructs thicker than a few millimetres, initial vascularisation from the surrounding host tissue is necessary.

It is difficult to reproduce the *in vivo* events completely *in vitro* using the basic knowledge of biology and medicine or cell culture technologies currently available. At present, it is difficult to realize *in vitro* tissue engineering because the artificial arrangement of a biological environment to induce cell-based tissue reconstruction is practically impossible. Even if a three-dimensional tissue-like construct is prepared *in vitro*, it is practically difficult for the construct to survive and function *in vivo* after grafting. In addition, the construct does not always connect with surrounding natural tissue biologically [324].

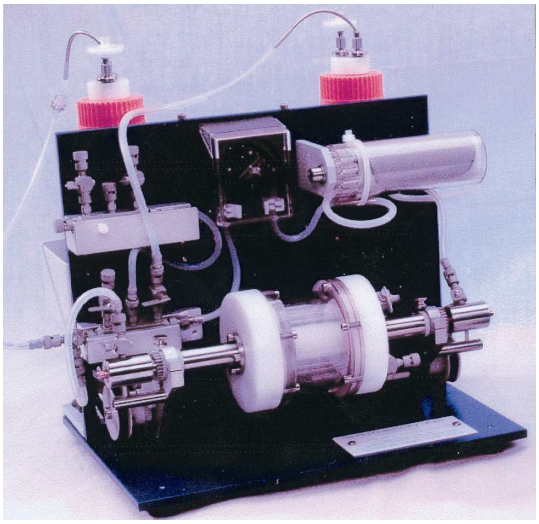

Figure 1.5: Example of a bioreactor for use in tissue engineering [99].

1.2.2.1 Mass-transfer requirements for tissue engineering

It has long been known that the supply of oxygen and soluble nutrients becomes critically limiting for the *in vitro* culture of three-dimensional tissues. The consequence of such a limitation is exemplified by early studies showing that cellular spheroids larger than 1 mm in diameter generally contain a hypoxic, necrotic centre, surrounded by a rim of viable cells [323]. Similar observations were reported for different cell types cultured on three-dimensional scaffolds under static conditions. Warburg [379] predicted many years ago that the maximal possible thickness achievable by diffusive transfer alone, with

blood as the non-circulating oxygen-carrying fluid, would be of 1mm for a cylinder-shaped tissue or complete developing organism, as calculated by the relation

$$r_{\max} = \sqrt{\frac{4Do_2BP}{Vo_2}}, \qquad \text{Equation 1.1}$$

Where Do_2B is the diffusion constant for oxygen in blood, VO_2 the rate of oxygen consumption in the tissue in question (for a human embryo, this rate is of approximately 6 mlO_2/min.kg), and P the oxygen partial pressure in the culture medium. It is interesting to note that, to date, the maximal thickness obtained experimentally in most cases is under this 1mm diffusive threshold [199]. To obtain tissue thickness clinically valuable, it must therefore be concluded that diffusive transport will have to be matched with convection to bring sufficient oxygen to the growing cells, whether an oxygen-carrying fluid is used or not.

Both for nutrient needs and waste elimination, mass transfer to and from tissues is a critical issue. *In vivo*, cells beneficiate from the proximity of blood capillaries for their mass-transfer requirements; in most tissues, cells are no more than 100 µm from these capillaries [365]. Thus, cells are only able to survive close to the surface. In this connection, it should be noted that no cell, except for chondrocytes, exists further than 25-100 µm away from a blood supply [112, 366]. Also, the small diameter of capillaries (6-8 µm) ensures a residence time long enough in tissues to permit the radial diffusion of chemical species [384]. Proteins and proteoglycans of the ECM generated by the cells are relatively large molecules and possess low intra-tissue diffusion coefficients, seriously hindering diffusion. For hepatocyte culture, it has been shown that when a reactor design relies solely on diffusion for the mass transfer of oxygen, cells must be within 150–200 µm of an oxygen source to survive and proliferate [203].

Table 1-2: O_2 solubility and consumption [104, 196]

	Typical culture medium	Pure water	
Atmospheric O_2 solubility (mmol/l)	0.2	0.4	
O_2 uptake (mmol/l) @10^6 cells/ml	Human skin fibroblast	Human liver cells	Blood
	0.064	0.30	up to 9.5mmol/l

Because engineered constructs should be at least a few mm in size to serve as grafts for tissue replacement, mass-transfer limitations represent one of the greatest challenges to be addressed. Oxygen is one of the most important nutrients for cells, being a major actor in all aerobic metabolic cycles. However, it is often the limiting nutrient in successful tissue growth *in vitro*. The reason for this arises from the difficulty of bringing sufficient amounts of oxygen to the surface of the cells mainly because of the poor solubility of oxygen in culture media (Table 1-2). Oxygen is typically consumed at approximately the same rate as glucose (on a molar basis), but oxygen solubility is lower than the availability of glucose (e.g., 20 mmol), for example. As a result, medium must be continually circulated and re-oxygenated by passing through an in-line gas exchanger. Moreover, an excess of oxygen in the medium surrounding the cells without an appropriate carrier such as haemoglobin, achieved by using pure oxygen instead of air or increasing gas pressure, induces the presence of free radicals, which are cytotoxic [98]. Indeed, hypo- and hyperoxic stresses have been implicated as causes of programmed cell death or apoptosis, which appears to be the main mode of cell death in many cultured cell lines [310]. However, cellular oxygen uptake varies depending on cell and tissue type (Table 1-2). Blood can carry more than 45 times the amount of oxygen that *in vitro* culture media can.

1.2.2.2 Limitations of tissue engineering scaffolds

The current inborn seeding-cells-on-scaffold regeneration method of engineered tissue is deficient for large constructs due to inadequate vascularisation [393, 394]. Several detailed investigations have shown that cells attach to synthetic polymer scaffolds leading to the formation of tissue [96]. However, the degradation of synthetic polymers, both *in vitro* and *in vivo* conditions, releases acidic by-products, which raise concerns that the scaffold microenvironment may not be ideal for tissue growth. Lactic acid is releases from poly(L-lactic acid) (PLLA) during degradation [270], reducing the p^H, which further accelerates the degradation rate due to autocatalysis [368], resulting in a highly acidic environment adjacent to the polymer. Such an environment may adversely affect cellular function. Cells attached to scaffolds are faced with several weeks of *in vitro* culturing before the tissue is suitable for implantation. During this period, even small p^H changes (p^H 6.8-7.5) in the scaffold microenvironment can significantly affect bone

marrow stromal cell expression of osteoblastic phenotypic markers. Furthermore, particles released during polymer degradation can affect bone-remodelling processes along with eliciting an inflammatory response and inducing bone resorption *in vivo*. Moreover, current synthetic polymers do not possess a surface chemistry which is familiar to cells, that *in vivo* thrive on an extracellular matrix made mostly of collagen, elastin, glycoproteins, proteoglycans, laminin and fibronectin [7]. In contrast, collagen is the major protein constituent of the extracellular matrix and is recognised by cells [159] as well as being chemotactic [261]. Collagen scaffolds presents a more native surface than to synthetic polymer scaffolds for tissue engineering purposes. However, like other natural polymers, it may elicit an immune response [18].

Table 1-3: Biodegradable polymers used for tissue engineering of cell scaffold and biosignalling molecule release.

Synthetic polymers	Natural polymers
poly(L-lactic acid) (PLLA)	collagen
poly(glycolic acid) (PGA)	gelatin
poly(e-caprolactone) (PCA)	fibrin
copoly(LL-GA)	hyaluronic acid *
copoly(LL-CA)	Alginate *
copoly(LLA-ethylene glycol (EG))	chitosan, chitin
copoly(fumarate-EG)	

* There are no enzymes in the body to directly degrade these polymers. They are washed out by body fluids to disappear from the implanted site.

Scaffold manufacturing techniques have improved a lot now and it is possible to manufacture scaffolds with finer diameter of elements. Even with the improvement, this approach has resulted in the *in vitro* growth of tissues with cross-sections of less than 500μm from the external surface [95, 140]. This is probably due to the diffusion constraints of the foam. The pioneering cells cannot migrate deep into the scaffold because of the lack of nutrients and oxygen and insufficient removal of waste products; cell colonisation at the scaffold periphery is consuming, or acting as an effective barrier to the diffusion of, oxygen and nutrients into the interior of the scaffold. Furthermore, for bone tissue engineering, the high rates of nutrient and oxygen transfer at the surface of the scaffold promote the mineralization of the scaffold surface, further limiting the mass transfer to the interior [198].

1.2.3 Alternative approaches

As discussed earlier, there had been limited success of current tissue engineering approaches with cartilage and skin; which can be explained in terms of cellular density, cellular energy demand and tissue thickness. Cartilage is relatively cell sparse, avascular tissue with relatively less energy consumption and nourished with diffusion from the surrounding tissue. Though skin is the largest organ of the body, it is relatively two-dimensional. For these reasons, construction of skin and cartilage is not much influenced by the mass transfer limitation of current tissue engineering approach. However, the scenario is quiet different for tissues rich in cells with more energy demand, like cardiac and hepatic tissue. The creation of 'thick' (>100–200 μm) cardiac patches has been limited by an inability to create the geometry necessary to support the high oxygen and energy demands of cardiomyocytes at a depth greater than ≈100 μm from the surface [85, 265]. This is actually the scenario in general for most of the tissue. Keeping these limitations in mind, there had been alternative attempt; like cell sheet engineering or use the nature's architecture- the extracellular matrix by decellularisation of tissue or organ.

The cell sheet engineering process used the temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm) to coat tissue culture polystyrene (TCPS) dishes, changing their property from hydrophobic to hydrophilic with change of temperature [302]. Normal TCPS dishes are hydrophobic and absorb extracellular matrix (ECM) proteins resulting in cell attachment and proliferation. The surfaces of PIPAAm are hydrophobic and cells adhere and proliferate under culture condition at 37° C. By lowering temperature below 32° C, the surfaces change reversibly to hydrophilic and not cell adhesive due to rapid hydration and swelling of the grafted PIPAAm. This unique surface change allows cultured cells to detach spontaneously from these grafted surfaces simply by lowering temperature. However, unlike trypsin treatment to harvest cells from culture dishes (which cause disruption to both adhesive proteins and membrane receptors and cells detach with considerable damages and cells are separated), this method allows harvesting cells in a single layer. These are then piled up to form a multilayer tissue like structure.

To use nature's architecture as a tissue engineering scaffold, the ECM of the donor tissue is prepared by the process of decellularisation. In this process, the cells from the

tissue are washed away keeping the tissue architecture intact. This is than repopulated with autologus cells from the recipient. This method has been used for transplantation of trachea in human [190, 191]. However, for other tissue like heart, it is still in the laboratory with no promising results [51, 68, 101, 103, 155, 244, 278]. It does not form real tissue rather a surface is coated with cells.

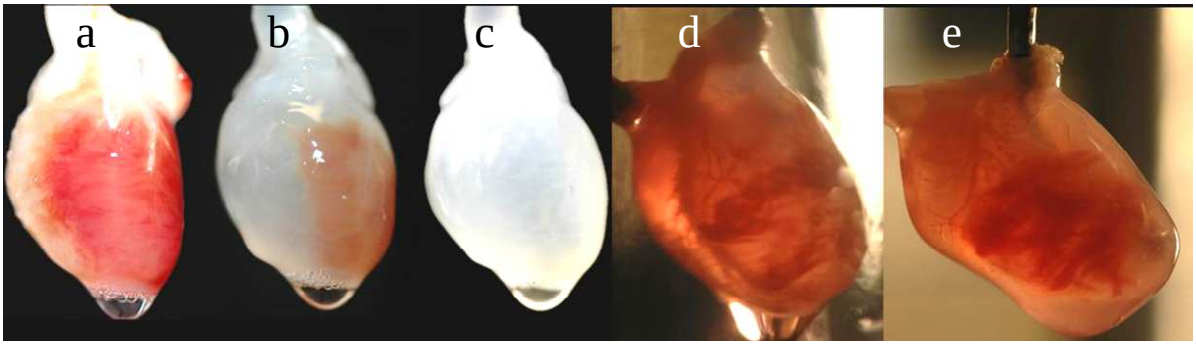

Figure 1.6: The use of nature's architecture as tissue engineering scaffold.
Rat heart decellularisation (a-c); and during recellularisation (d-e) [201].

1.3 Embryogenesis in comparison with *in vitro* culture of cells

The cultivation of anchorage-dependent animal cells is performed by using solid cell-culture substrates, such as polystyrene dishes, microcarriers and macroporous carriers where the whole process is governed by the physiochemical properties of the solid substrate. Cells are cultured at the interface between solid substrate and liquid culture medium. Rather than forming an organised three-dimensional tissue-like structure, cells divide and grow in a single layer following the geometry of the substrate. Cellular phenotype is often dominated and limited by the cellular microenvironment and technical boundary conditions of the substrate. Inappropriate alteration to cellular microenvironment and rigid substrates hinder cellular communication, cell division, differentiation and migration. This results into a flat layer of homogenous cell sheet, which is unlike tissue consisting of inhomogeneous cells performing a specific function in coordination. The current technology of *in vitro* culture in flasks or on dishes developed from Petri dishes and nutrient-gel-surface culture of microbiology. It is less automated, allows insufficiently defined microenvironment of cells and limited and dominated by technical boundary conditions of the flask. In general, these methods are used to keep and culture cells outside the organism.

In contrast, the process of embryogenesis takes place at the transition zone of two liquid substrates. As observed in avian embryo, the embryonic disc (also called the blastoderm, the blastodisc, the disc of blastomeres) is the mass of cells that lies at the interface of egg yolk and egg white during the time of lay. With the incubation, these cells grow and start to differentiate. Here the cells are not guided or dominated any solid surface. During embryogenesis, dominated by cell microenvironment and orderly cell migration in groups – from the germ layer, forms the tissue, organ and at last forms a functional organism. Apparently, this three-dimensional freedom of movement facilitates cell division and migration.

In chicken, the oocytes accumulate so much yolk in its cytoplasm that the nucleus of the cell and most of the cytoplasm contents (particularly those needed by the nucleus to divide) are pushed to one end of the cell. The yolk contains all the nutrients needed by a growing embryo. A non-cellular vitelline membrane produced by the ovum surrounds the cell membrane of the ovum. Cell division begins soon after fertilization. As the yolk passes through the oviduct, the yolk becomes invested with several layers of albumin and the cells of the germ disc lies at the interface. By the time the egg is laid, the embryo has reached the blastoderm stage where the initial development of the embryo takes place. Several thousand cells form two layers (epiblast and hypoblast) called "gastrula." The embryonic disc arose by meroblastic cleavage of the germ disc. Cleavage is incomplete (meroblastic) and is restricted to the small portion of yolk-free cytoplasm called the germinal disc. At this time the egg is laid, it cools, and embryonic development usually stops until proper environmental conditions are established for incubation.

With the proper environmen (incubation), the process of development resumes. The avian egg as a culture system has a dynamic and permanent changing boundary conditions, which adapts to the changing need of the growing embryo. *In vitro* culture of cells, on the contrary, has static boundary conditions all the time. This is a very important difference in this regard especially concerning stem cell differentiation where the differentiated cells may have different metabolic status and microenvironment then in the undifferentiated status. However, cells are cultured in the same boundary conditions. This is no surprise that differentiated cells will have altered physiology in the unchanged boundary conditions.

As the process of embryonic development continues, a furrow (primitive streak) appears in the midline of the embryonic disk which is formed by the ingression of epiblast cells which will go on to form the definitive endoderm and mesoderm by replacing hypoblast cells. The epiblast cells then develop into the definitive ectoderm. Gradually, a highly vascularised membrane begins to appear on the yolk and by the end of 4th day of incubation, it completely surrounds the yolk. At this stage the contents of the yolk is compartmentalised with the yolk enclosed by the yolk sac, which is further enclosed by egg white. The embryo lies at the interface over the yolk and egg white over the vascular yolk sac. Turning of eggs by the incubating bird during the process of embryonic development may have determinative effect on the embryonic development where the embryos from unturned eggs fail to hatch. As the development proceeds, a fluid filled sac called amniotic cavity surrounds the embryo and the CAM is formed on the fourth day of incubation by the fusion of the ectodermal epithelium (chorion) and the endodermal epithelium (allantois). At this stage, undifferentiated blood vessels are scattered in the mesoderm of the CAM. These vessels grow rapidly until day 8, when some vessels differentiate into capillaries and form a layer at the base of the ectoderm. At ID 14, 6 days before hatching, the capillary plexus is located at the surface of the ectoderm adjacent to the shell membrane [282]. This extraembryonic membrane serves as a transient gas exchange surface similar to the lung. An extensive capillary network provides its respiratory function. The CAM functions for gas exchange (lung), storage of excretory urinary products (reservoir function), and mobilization of calcium from the shell to start bone mineralization (mineral resorption).

1.4 The chicken embryo – a model system

The embryo of *Gallus gallus domesticus*, the chicken, has a long history as a model system in developmental biology and has contributed major concepts to immunology, genetics, virology, cancer and cell biology [313]. The chicken egg is such a common and easily accessible source for embryological studies that it attracted attention from the ancient Egyptians as well as the Greek philosopher Aristotle, who opened eggs at different stages of incubation to examine developmental progress. Important discoveries that have been made in the chicken embryo include the function of arteries and veins [125], the existence of capillaries [194, 195], the existence of the neural tube [195], and the

derivation of organs from the germ layers (ectoderm, endoderm and mesoderm) and from mesenchyme such as the neural crest [249]. It was demonstrated first in the chicken embryo that viruses can cause cancer (Rous Sarcoma virus) [286], that extraembryonic endoderm (hypoblast) regulates embryo polarity [313], that the somites control the segmentation of the nervous system [156], and that the notochord patterns the dorsal-ventral axis of the spinal cord [364, 395]. The hemangioblast, common precursor of endothelium and blood cells, was first demonstrated in the chicken embryo [69], T- and B-lymphocytes were first described in the chicken embryo [213, 313], and the first cellular onocgen (c-src) was described in the chicken embryo [367], to name a few of the many important discoveries made in this remarkable model system. At early stages of development, the chicken embryo has no immune system, and can therefore serve as a host for tissue implantation, both conspecific and xenotypic, without rejection. For example, transplantation of chicken and quail tissue has been used as an embryological tool for decades, and in more recent years xenotypic transplantation of mouse embryonic tissue to chicken embryos has become a popular means of studying mouse tissue development *ex utero* [181, 260, 389]. The implantation of human and mouse stem cells into chicken embryos is more recent but has been shown to yield important new discoveries [92, 106, 219, 260, 385, 389]. The use of chicken embryos in SC research has great potential, not just for studying endogenous stem and progenitor cells during development, but also as a transplant model for studying the in vivo properties of various types of stem and progenitor cells.

1.4.1 The chicken egg – a model system to promote *in vitro* cell culture as well as tissue engineering

Embryonic development is a tremendously complex process, which has fascinated man since the beginning of history. How does fertilization result in the formation of a complete, independent individual? Where is the information for this complexity encoded, and what mechanisms ensure that it is decoded appropriately? To answer these fundamental questions, science has made use of a number of "model systems," each with different advantages in that they allow various experimental approaches to different extents.

The most important metazoan model systems for studying development currently include the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, a few species of sea urchin (mainly *Strongylocentrotus purpuratus* and *Lytechinus variegatus*), the zebrafish *Danio rerio*, the South African clawed toad frog *Xenopus laevis*, the chicken *Gallus gallus domesticus*, and the mouse *Mus musculus*. Of these, the chicken was the first to be used for developmental investigations.

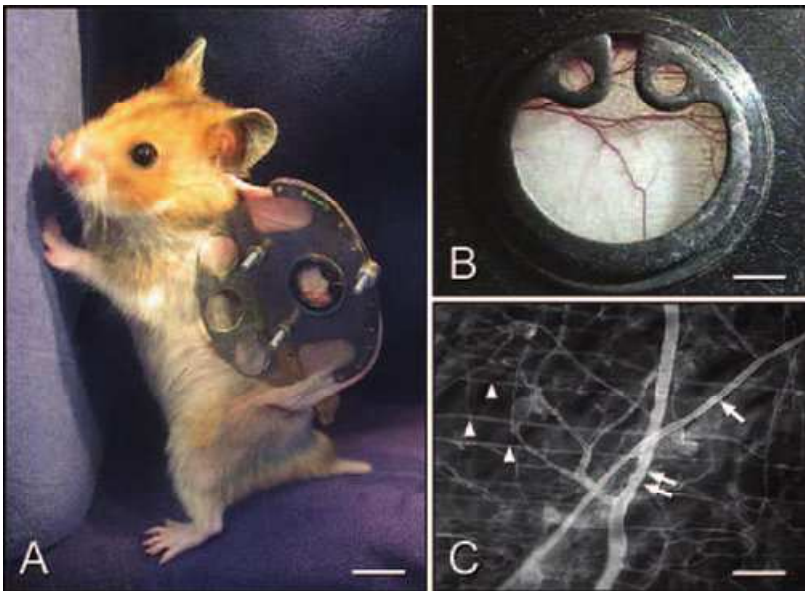

Figure 1.7: Tissue-engineering models.

(A) Syrian golden hamster equipped with a dorsal skinfold chamber (weight ≈4 g). (B) Overview of the observation window after the implantation of the chamber into the dorsal skinfold. (C) Intravital fluorescence microscopy of the micro-angioarchitecture of the dorsal skinfold chamber consisting of arterioles (arrow), parallelly arranged muscle capillaries (arrowheads), and postcapillary and collecting venules (double arrows). Scale bars: A = 12.5 mm; B = 2.5 mm; C = 105 µm [178].

To improve current techniques and to develop new strategies for optimal vascularisation of implanted tissue constructs, sophisticated experimental models are required that allow for a detailed analysis of blood vessel ingrowth in engineered tissue constructs *in vivo*. The generation of 3D tissues *ex vivo* not only requires the development of new biological models for traditional monolayer or micromass cell cultures [62], but also poses new technical challenges owing to the physicochemical requirements of large cell-masses. Previous studies have analyzed the process of angiogenesis primarily using *in vitro* cell culture experiments and histological examinations of formalin-fixed tissue. Recently, however, two commonly used *in vivo* models to study angiogenesis, the CAM assay and the dorsal skinfold chamber, have

been introduced in the field of tissue engineering. Since, now animal experiments are discouraged, the CAM of the developing chicken embryo offers advantages over the other model system.

Lack of a functional immune system of developing chicken embryo before embryonic Incubation Day (ID) 17 of prevents transplant rejection [200]. Different applications of the CAM model in areas of interest for the pharmaceutical community, such as angiogenesis and antiangiogenesis [273, 275, 277], wound healing [274], tissue engineering [32], biomaterials and implants [163, 362, 402] and biosensors [361]. Three extraembryonic membranes protecting and nourishing the embryo are formed during development: the yolk sac membrane, the amnion, and the CAM. The latter is a transparent and highly vascularised membrane, formed during the ID 4 to 5 by the fusion of the mesodermal layers of both the allantois and the chorion, resulting in a highly vascularised mesoderm composed of arteries, veins, and an intricate capillary plexus [273, 291].

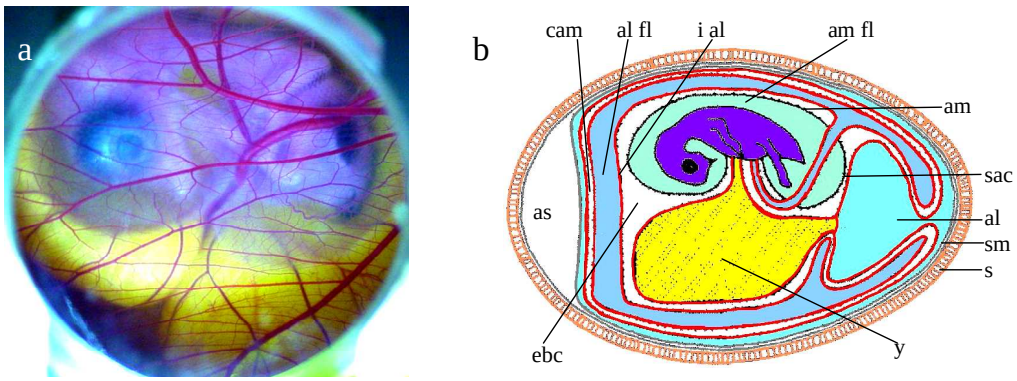

Figure 1.8: Chick embryo Chorio-Alantoic Membrane (CAM).

(a) CAM image taken from the top of the open culture system at ID 12. (b) Development of the domestic fowl egg: after ID 12. s, shell; sm, shell membrane; as, air space; y, yolk; al, albumen; sac, sero-aminiotic connection; am, amnion; ebc, extra embryonic body cavity; cam, ChorioAllantoic Membrane; i al, inner allantois; al fl, allantoic fluid; am fl, amniotic fluid (redrawn after Burton et al, 1985 [43]).

This model has also been used as a short-term *in vivo* system in numerous studies in a wide range of fields: human skin grafting [172], liver [149] and skeletal muscle regeneration [225], surgical retinal research and simulation [184], tissue responses to biomaterials [33, 362], photodynamic therapy [175], as an alternative to the Draize rabbit eye irritation test [37], grafting of mammalian cells and tissues to the embryonic chick [148] and is extremely accessible to experimental manipulation..

The CAM, is a well-established experimental system for studying solid tumours, embryonic development, and evaluating angiogenic and antiangiogenic drugs. The CAM is a respiratory organ composed of several layers, a low stratified epithelium positioned externally, adjacent to the shell membrane, and an underlying dense plexus of capillaries where gas exchange takes place. Internally, lining the amnion, there is a simple cuboidal epithelium. In between the outer and inner epithelia lies a highly branched plexus of blood vessels of many sizes, the largest of which are accompanied by a dense plexus of lymphatics. The large vessels are embedded in loose mesenchyme. The ability of cells to from solid cancers grafted to the CAM to reproduce many of the characteristics of tumours *in vivo*, including tumour mass formation, angiogenesis, and metastasis, has been utilized in many studies

The extracellular matrix constitution of CAM is similar to peritoneum [200]. The best current *in vivo* models for studying human blood malignancies and leukaemia therapy are highly immune-deficient mice or foetuses of large mammals (sheep and dogs) in which hematopoietic cells are capable of proliferation [48, 325]. These large-animal models are not practical for studying the biology of blood malignancies or for drug screening, because of the high cost and large space requirements for the experiments. Although the immune-deficient mouse models are powerful and recapitulate the phenotypes of blood malignancies *in vivo*, the purchase price per mouse is high, the maintenance of these mice is costly and complex, and more than 1 month is required to establish engraftment.

1.4.2 Historical studies on chicken eggs

The embryo of the domestic fowl (*Gallus gallus domesticus*) holds the record as the animal with the longest continuous history as an experimental model for studies in developmental biology, spanning more than two millennia. Throughout this time, it attracted great naturalists, artists, philosophers, and pioneers of biology and stimulated them to think about the most fundamental questions on generation and life. The ancient Egyptians are documented as having opened hen's eggs at different periods during incubation to observe the progress of embryonic development. By around 300 BC Aristotle undertook careful studies of the morphology of the embryo (as much as he could without the aid of magnifying devices); this can be considered as the first

'scientific' study of embryo development and his work referred to by his followers right up to the 19th century. After the mediaeval **'Dark Ages'**, the resurgence of an interest in anatomy and embryo development in the Renaissance attracted figures including Leonardo Da Vinci (1452–1519), Ulisse Aldrovandi (1522–1605) and Hieronymus Fabricius ab Aquapendente (1537–1619) to return to the study of the embryo within the egg.

Until well into the 19th century, observations of chicken embryos at different stages were used to support either of the two theories of the raging debate between preformation (the adult is preformed in miniature from the time of fertilization or even earlier, and just grows) and epigenesis (the embryo increases in complexity and new organs form as it develops) [235, 312, 391]. Along the way, the philosophers made many discoveries, as important as blood islands and the functional difference between arteries and veins, which were proposed to be connected to each other by capillary vessels [125]. William Harvey (1578–1657) observed chick embryos at early stages of development and concluded that the heart was the first functioning organ to develop in the embryo. By observing the motion of the blood through the heart and early vessels, he discovered the circulation of the blood and understood the function of arteries and veins. The existence of the capillary was later confirmed with the aid of a simple microscope by Malpighi, who also discovered (despite his preformationist convictions) the existence of the neural groove (neural tube) and the somites and that the beating of the heart began even before the blood started to form [194, 195]. Subsequent progress closely followed new technical advances. Improved microscopes and early attempts at sectioning allowed the discovery of the germ layers [249, 369] and the first indications of interactions between them, which later led to the concept of induction. The introduction of histological sectioning and of selective staining methods allowed Pander and von Baer to start to understand the significance of germ layers in development. These pioneers also started to ask questions about causality in development—what mechanisms are responsible for such stereotyped development?

After the mid-1800s, the new innovation was the introduction of numerous selective dyes for staining and more sophisticated methods for sectioning, which sprouted a new generation of comparative histologists (mainly in Germany, including von Ebner, Hensen, Rauber, Koller, and Remak) who quickly generated a comprehensive

description of the changes in structure of the embryo throughout development. Many of the modern concepts and the names of anatomical components of the embryo are due to the work of these pioneers, whose keen powers of observation combined with their curiosity to establish the first mechanistic insights into how development might occur. Further and increasingly careful histological studies followed throughout the 19th Century, with the most important contributions being made by Rauber and Hensen and culminating in a beautiful histological atlas by Mathias Duval (1889).

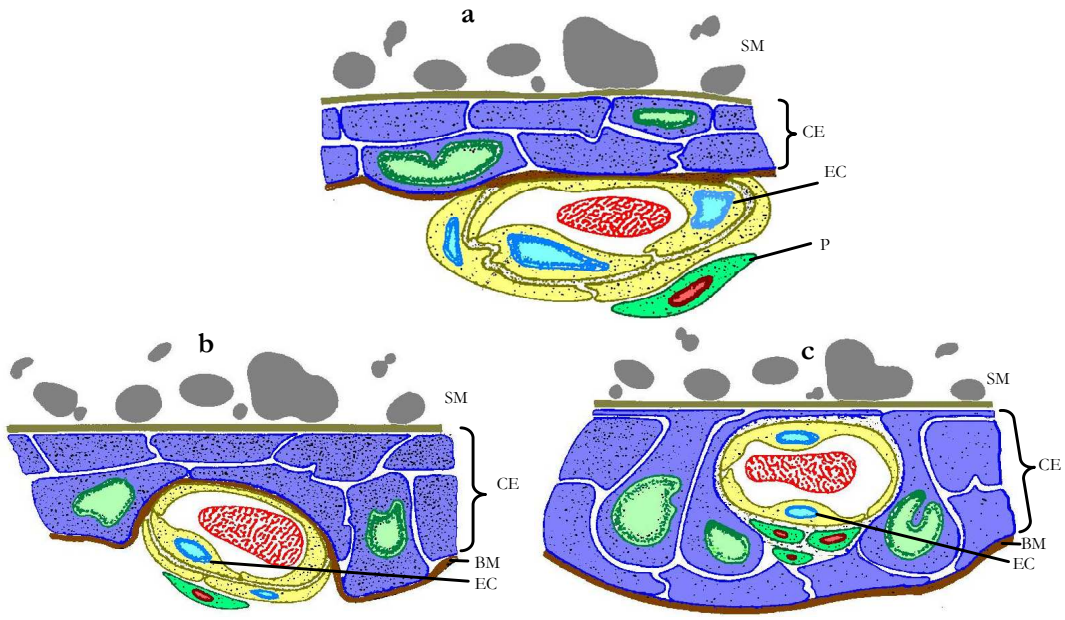

Figure 1.9: Structural reorganization of the CAM between ID 8 and ID 12.

Structure of the CAM of a chick embryo on (a) ID 8, (b) ID 10, and (c) ID 12. Shown is development from subepithelial (a), intraepithelial (b), to supraepithelial (c) vascularisation of the CAM. CE, Chorion epithelium; EC, endothelial cell type of capillary; P, pericyte; SM, shell membrane; BM, basal membrane. (redrawn after Fitze-Gschwind, 1973) [89].

By the end of the 19th century, embryology was born again. Wilhelm Roux and his followers realized that carefully designed experimental manipulations that disturb development could provide information about the developmental potential of cells in the embryo, far beyond the speculations that had previously been attached to static histological observations. These studies were quickly applied to many species and led to detailed fate maps, formal definition of concepts such as regulation, induction, commitment, and competence, and the clear notion that development depends upon the flow of instructive signals between different cell populations.

Around the same time (ca. 1910), Thomas Hunt Morgan was building the discipline of developmental genetics and introducing the fruit fly as a system—the combination of Roux's **"Experimental Embryology" (Entwicklungsmechanik)** with Morgan's genetic analysis signalled the birth of modern developmental biology. At that time, experimental embryology (Entwicklungsmechanik) started to replace simple histological observation as it became clear that principles could only emerge from experimental manipulation of the embryo. However, the initial advances were mainly made through work in other organisms (sea urchins and amphibians for "embryoembryonic regulation" and induction, marine invertebrates for lineage studies, Drosophila for developmental genetics) and the chick was a little slower in catching up. But there were some salient chick studies at this time. Which include Graeper's spectacular three dimensional stereo time-lapse movies of embryos labelled with spots of vital dyes to follow cell movements (made in 1926, published in 1929 and unrivalled to the present day), which revealed the cell movements preceding and during gastrulation and Waddington's cross-species transplants of primitive streak and node and his hypoblast rotations which led to the first evidence that extraembryonic endoderm (hypoblast) plays a role in positioning the embryonic axis [311], as well as consolidating the concept that Hensen's node is a source of signals for neural induction in both mammals and birds. Likewise it is not widely known that Waddington pioneered an experimental approach to understanding the development of left–right asymmetry [372, 373].

In the last 50 years, the chick embryo has contributed some of the most important general concepts in vertebrate developmental biology. This includes the discovery of the mechanisms that pattern the vertebrate limb and the ZPA and AER as signalling regions therein (John Saunders, Lewis Wolpert, Cheryll Tickle), the demonstration of the movements and fates of the neural crest by Le Douarin, the discovery that the notochord (and Sonic hedgehog signalling from it) regulates dorsoventral polarity and the location of different neuronal subpopulations within the neural tube by van Straaten and Jessell, the importance of somites in controlling segmentation of the peripheral nervous system (Keynes and Stern) while the central nervous system is autonomously segmented (Lumsden), the discovery of T- and B-cells and the hemangioblast by Le Douarin and colleagues, and many more. As molecular biology merged into

developmental biology, it was in the chick that the first 'dynamic' gene expression pattern (Hairy-1 and Lunatic Fringe) was discovered to be correlated with somite formation (Pourquie) and that the first four genes regulating left–right asymmetry were found (Sonic hedgehog, Nodal, Activin-receptor IIA and HNF3b) [186]. Moreover, the DT40 cell line has also turned out to be a superb system to study genetic recombination and the origin of immunological diversity.

Table 1-4: Some Major Concepts due to work on Chick Embryos

Date	Concept	Discoverer(s)
1628	function of arteries and veins, proposed existence of capillaries	Harvey
1672–1675	neural tube, somites, capillaries	Malpighi
1817–1828	germ layers (ectoderm, mesoderm, endoderm)	Pander, von Baer
1868	the neural crest	His
1911	viruses cause cancer (Rous Sarcoma Virus)	Rous
1929	gastrulation cell movements (Polonaise)	Gräper, Wetzel
1932	extraembryonic endoderm (hypoblast) regulates embryo polarity/mesoderm induction	Waddington
1932		Murray
1932–1937	hemangioblast proposed (common precursor of endothelium and blood cells)	Waddington
1936	Hensen's node is the amniote organizer	Hutt
1948–1968	first genetic map for the chicken	Saunders
1953	Apical Ectodermal Ridge controls limb outgrowth	Bellairs
1956	gut endoderm is derived from the epiblast via the primitive streak	Zwilling, Saunders
1960–1968	Zone of Polarizing Activity patterns the A/P axis of the limb	Miller, Good, Glick, Claman
1964–1970	T- and B-lymphocytes	Temin
1967	provirus hypothesis and reverse transcriptase	Abercrombie
1970	contact inhibition	Eyal-Giladi and Wolk
1975	importance of extraembryonic endoderm (hypoblast) in head development	Dieterlen-Lie` vre, Le Douarin
onwards	hemangioblast demonstrated	Bishop and Varmus
1976	first cellular oncogene (c-src)	Keynes and Stern
1984	somites control segmentation of peripheral nervous system	Tickle, Eichele
1985–1987	retinoic acid as a limb morphogen	Van Straaten
1988	the notochord patterns the dorsoventral axis of the spinal cord	Lumsden and Keynes
1989	rhombomeres are embryologically and functionally important	Buerstedde
1991	DT40 cells undergo frequent homologous recombination	Jessell
1993	Sonic hedgehog patterns the spinal cord (D/V) and specifies motor neurons	Tabin
1993	Sonic hedgehog is the ZPA morphogen	Tabin
1995	a genetic cascade patterns the dorsoventral axis of the limb	Tabin, Kuehn, Stern
1995	a genetic cascade regulating left-right asymmetry	Pourquie´
1997	oscillating gene expression during somitogenesis	

It seems extraordinary that despite such a history, in more recent days there have been signs of 'anti-chick' racism by institutions hiring new young faculty. this animal possesses about the same number of genes as humans but is extremely compact and with an amazing level of conserved synteny with mammals [312]. In the last few years, the classical approaches have been enormously enriched by three major technical advances: the introduction of new methods for gain- and loss-of-function and promoter analysis, the isolation of embryonic stem cells and development of new methods for transgenesis, and the sequencing of the chicken genome and establishment of numerous new electronic resources [313]. These new technical advances promise to give the chick embryo

a huge new impetus as a leading system for developmental biology and many other areas.

1.5 The physiochemical basis of the chicken egg model: liquid│liquid interface culture system

From a technical and biotechnological perspective, the avian egg can be seen as an interface of two immiscible liquids, where the blastoderm develops at the interface between a protein rich in water (egg white/albumen) and lipid (egg yolk). In the conventional cultivation, cells grow on some solid substrates as a monolayer, most commonly glass, or non-toxic plastic in contact with tissue culture medium. Typically, the substrate may be the inner surface of the vessel containing the medium or may be the surface of small solid particle kept suspended in the medium (microcarrier culture). In contrast, the process of embryogenesis takes place at the transition zone of two fluids (as observed in avian embryogenesis where the blastoderm is located between the egg white and yolk). As in nature, cells can also be cultured at the liquid│liquid interface. Two immiscible liquids placed together separate from each other creating a liquid│liquid interface. One of these liquids can be tissue culture medium and other liquid, relatively immiscible with the first one, is hydrophobic, higher density than water and non-toxic to living calls. In this cultivation method, anchorage dependent animal cells can anchor, spread and grow at the interface [151-153]. As is the case of solid substrate, the cells do not directly interact with the interface, but rather the proteins adsorb to the interfacial junction.

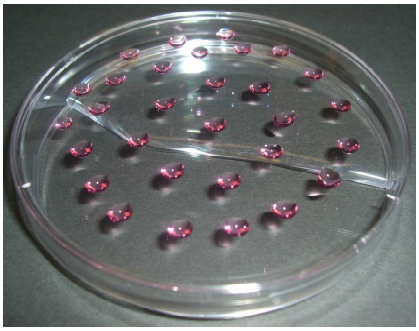

Figure 1.10: Example of interface culture: hanging-drop culture.
Cells are cultured at the liquid│gas interface.

Consider an interfacial surface S bound by a closed contour C (Figure 1.11). One may think of there being a force per unit length of magnitude σ in the s-direction at every point along C that acts to flatten the surface S.

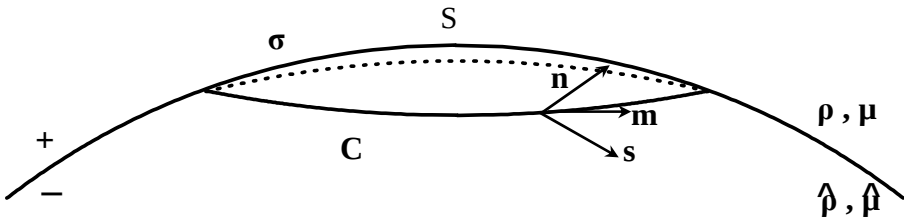

Figure 1.11: A surface S and bounding contour C on an interface between two fluids.

The upper fluid (+) has density ρ and viscosity μ; the lower fluid (-), $\hat{\rho}$ and $\hat{\mu}$. n represents the unit outward normal to the surface, and $\hat{\mathrm{n}} = -\mathrm{n}$ the unit inward normal. m the unit tangent to the contour C and s the unit vector normal to C but tangent to S.

Perform a force balance on a volume element V enclosing the interfacial surface S defined by the contour C:

$$\int_V \rho \frac{D\mathrm{u}}{Dt}\, dV = \int_V f\, dV + \int_S \left[\mathrm{t(n)} + \hat{\mathrm{t}}(\hat{\mathrm{n}})\right] dS + \int_C \sigma \mathrm{s}\, d\ell \quad , \qquad \text{Equation 1.2}$$

Here ℓ indicates arclength and so $d\ell$ a length increment along the curve C. $\mathrm{t(n)} = n \cdot \mathrm{T}$ is the stress vector, the force/area exerted by the upper (+) fluid on the interface. The stress tensor is defined in terms of the local fluid pressure and velocity field as $\mathrm{T} = -\mathrm{p}\,\mathrm{I} + \mu\left[\nabla \mathrm{u} + (\nabla \mathrm{u})^T\right]$. Similarly, the stress exerted on the interface by the lower (-) fluid is $\hat{\mathrm{t}}(\hat{\mathrm{n}}) = \hat{\mathrm{n}} \cdot \hat{\mathrm{T}} = -\,\mathrm{n} \cdot \hat{\mathrm{T}}$ where $\hat{\mathrm{T}} = -\hat{p}\,\mathrm{I} + \hat{\mu}\left[\nabla \hat{\mathrm{u}} + (\nabla \hat{\mathrm{u}})^T\right]$.

Physical interpretation of terms

$\int_V \rho \frac{Du}{Dt} dV$: inertial force associated with acceleration of fluid within V

$\int_V f dV$: body forces acting on fluid within V

$\int_S t(n) dS$: hydrodynamic force exerted at interface by fluid +

$\int_S \hat{t}(\hat{n})\, dS$: hydrodynamic force exerted at interface by fluid -

$\int_C \sigma s d\ell$: surface tension force exerted along perimeter C

In 1964 Rosenberg introduced the use of a fluid substrate for the growth of both transformed and anchorage-dependent cells [284, 285]. In this method, a cell suspension is introduced over an inert hydrophobic liquid having a density greater than that of the aqueous medium, and cells are observed to spread and divide on the liquid | liquid interface between the two immiscible phases. As is the case for solid substrates, the cells do not interact directly with the interface but rather with proteins that adsorb to the interfacial junction. These proteins presumably denature as their polypeptide chains unfold to achieve a low energy orientation with most of the hydrophilic portions exposed to the aqueous phase and the hydrophobic portions in the inert nonaqueous phase. One of these liquids can be tissue culture medium and other liquid, relatively immiscible with the first one, is hydrophobic, higher density than water and non-toxic to living calls. In this cultivation method, anchorage dependent animal cells can anchor, spread and grow at the interface [151-153]. Different membranes can be placed at the interfacial zone, which may include natural and synthetic membranes, micro and nano structures and biomatrices.

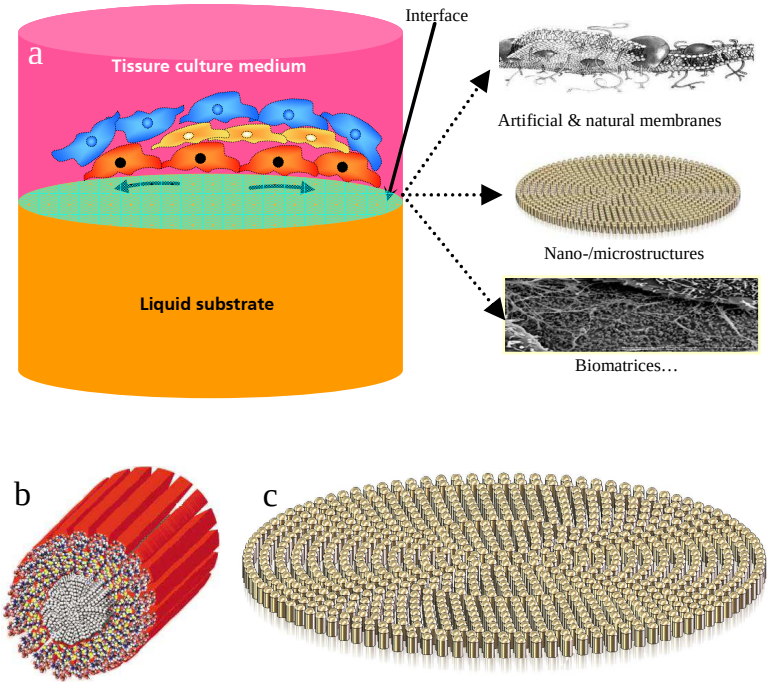

Figure 1.12: Principle of liquid | liquid interface cell culture system

(a) Cells are cultured at the interface between two fluids. Different materials can be used at the interfacial zone as culture substrates like different artificial and natural membrane, nano/micro structures and biomaterials. (b) [1] and (c) self assembling nanofibres.

Cells grown on such substrates can be transferred by simply pipetting the cell layer. Such a procedure of cell harvesting is especially important where the effect of trypsin or other proteolytic enzymes or chelating agents to passage cultured cells should be avoided. The compositions of cell membrane proteins are changed by enzymatic treatment or by mechanical scraping [301]. Such a procedure is of particular interest in that it obviates the use of trypsin or other proteolytic enzymes or chelating agents to passage cultured cells and avoids the uncertain effects of such treatments. If the adsorbed serum proteins at the interface are crosslinked by glutaraldehyde or if a bimolecular layer of proteins is formed by using polylysine as a base coat [88], the patterns of cell growth can be altered significantly. This simply means that cell growth can be regulated as desired. In addition this method is free from the influence of the technical boundary conditions of the substrate and allows freedom of cellular migration similar to the developing avian embryo at the interface of egg white and egg yolk.

There are different materials can be used as liquid substrate for liquid|liquid interface culture. Among them perfluorochemicals and silicone oil are most noticeable. Flurocarcons are non-toxic, inert, higher density (specific gravity 1.9), immiscible with water, low viscosity, thermally and chemically stable, optically transparent, hydrophobic and especially high solubility for gases. The fluorocarbon|aqueous interface is well suited to provide an inert, nontoxic, hydrophobic substrate for cell growth. It has the advantage of being exceptionally homogeneous and reproducible when compared with hydrophobic solid surfaces, which, in general, have polar molecular inhomogeneities.

One other special property of the avian eggs as a culture system is the dynamic and permanent changing boundary conditions, which changes permanently in accordance with the changing need of the growing and differentiating calls as well as the embryo. Traditional *in vitro* culture of cells, on the contrary, uses static boundary conditions for culturing cells. The differentiated cells may have different metabolic status and microenvironment then in the undifferentiated state. However, cells are cultured with static boundary conditions. It is no surprise that the differentiated cells will have altered physiology in the unchanged boundary conditions.

2 Objective of the study

The surface culture of cells is far different from the three-dimensional case *in vivo*. One reason lies in the equipment and the method of *in vitro* culture of eukaryotic cells. The basic principle of the surface culture is actually adapted form nutrient-gel and dish culture of Microbiology, which is actually designed for prokaryotic cells. In general, these methods are used to culture cells outside the organism. Unlike *in vivo*, cells there act as independent units, much like a microorganism such as a bacteria or fungus. Cell growth is governed mainly by the geometry and surface property of the solid substrate. In many cases, the appropriate biological, physical and chemical cues are not yet completely understood. The ability to control differentiation of stem cells into specialized cell types with high yield and precision is a key success factor that will determine the ultimate utility of such research. However, researchers are facing significant challenges in these efforts because stem cells are difficult to handle and there are very few automated or standardized tools available in this relatively new field.

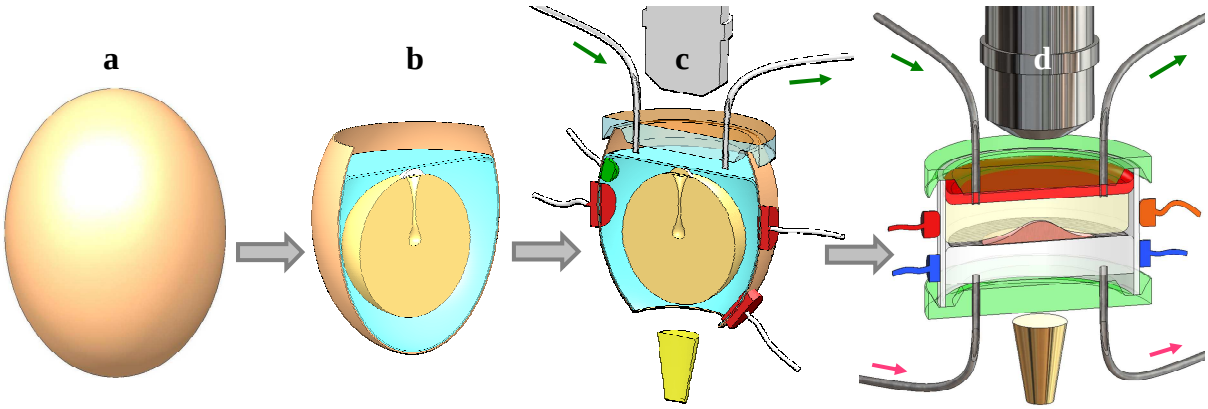

Figure 2.1: Derivation of liquid | liquid interface culture (computer simulation).

The open system of avian culture will be technically modified step by step and at the end a complete artificial system will be developed to culture mammalian cells which will contain no embryo.
(a) Chicken egg; (b) surrogate shell open culture system; (c) technically modified surrogate shell open culture system optimized for optical imaging, electrodes and sensors implanted for different bioelectrical signal acquisition; (d) complete artificial system for *in vitro* cell culture which contains no chicken embryo at the end but cell culture adopt the same principle. Green arrow = channels for culture medium exchange; Pink arrow = channels for substrate exchange. Note, at the end the system will contain different sensors to monitor culture environment and the whole system will be mounted under especially constructed microscope to image at cellular level.

The avian egg, in all its complexity, is still a mystery. Unlike mammals, it is separated from the mother animal contains everything for the growth and development of a multicellular organism from single fertilized oocytes whose cells and tissues have adopted differentiated characteristics or fates to perform the specified functions of each organ of the body. It is able to maintain the exact microenvironment necessary for the dividing and differentiated cells as well as the growing embryo all by itself. This remarkable feature had its perfection throughout the evolution of millions of years. From biotechnological and technical point of view, the avian egg can be considered as a construction plan for new *in vitro* culture system since it is independent of the mother animal and self equipped.

In birds eye view, the chicken egg looks very simple, having only two components– the egg white and the yolk. However, it is a highly complex reproductive cell-essentially a tiny centre of life. Because of its independency and completeness, it is much easy to observe and study the vital process at the beginning of life at the cellular level in avian egg than in the mammals. It has been suggested that tissue neogenesis in tissue engineered constructs may involve the same processes present in tissue development during embryogenesis. This is supported by studies in which periosteal cells on a polyglycolic acid scaffold appeared to first generate hypertrophic cartilage prior to mineralized bone formation [357] as *in vitro* situation where cartilaginous centre of ossification appears first which ossifies to bone later stage. The systems biology approach to developmental biology emphasizes the importance of investigating how developmental mechanisms interact to produce predictable patterns (morphogenesis). Careful *in vivo* observation at cellular level may reveal the secret of organised cell migration, cell communication, cell differentiation, tissue formation, organogenesis and the development of the whole organism. A better understanding of normal cell development will allow understanding and perhaps correcting the errors that cause these medical conditions. Studying stem cells will help us understand how they transform into the dazzling array of specialized cells that make a complete animal.

The objective of the thesis is to initiate a bridge between developmental biology and cell biology/stem cell research, to make a platform to study the process of morphogenesis, organogenesis, as a whole embryogenesis. So that using this ground further works can be done in future to develop a bioreactor with more flexible and

physiological cell culture conditions avoiding the disadvantages of conventional solid surface cell culture system with adjustable boundary conditions; to develop an *in vitro* tool for exact cell differentiation comparable to embryogenesis. This new approach may open a new horizon in the field of *in vitro* cell culture as well as for tissue engineering. The idea behind this thesis is to make gradual technical modifications of the avian egg and gradually adapt it for *in vitro* cell culture. Based on this functional biological system, alternative artificial *in vitro* culture systems should be tested in accordance with embryogenesis. Here is the important point of interest, the induced linage specific differentiation of stem cells *in ovo* to provide the basic technology for the routine cell culture as well as the observation and documentation at or near the cellular resolution. The real-time acquisition of dynamic process will be possible through time lapse and slow motion characterization e.g. of cell migration.

At the heart lies the avian egg with different technical modifications: with the change of forms, access for optical observations, installation of sensors to access biosignals and high resolution imaging. Preliminary works show (Chapter 4) that, this is possible, since it is possible to demonstrate the complete development of avian embryo (from the blastoderm stage to viable hatchlings) in a complete open system of *in vitro/ex ovo* culture or in a complete artificial system. It is the hatching of a viable bird, which is regarded as the milestone for the development of a new liquid│liquid interface *in vitro* culture system. Human stem cell research holds enormous potential for contributing to our understanding of fundamental human biology. Although it is not possible to predict the outcomes from basic research, such studies will offer a real possibility for treatments and ultimately for cures for many diseases for which adequate therapies do not exist.

In this contextthere are three parallel investigations

- Culture of animal and human cells in unfertilized and fertilized egg,
- Iterative introduction of technical components to the open system of avian culture,
- Development and testing of complete artificial liquid│liquid interface culture system through gradual removal of egg components (e.g. egg shell)

At first, the liquid│liquid interface of unfertilized or fertilized avian eggs will be used to culture cells. There the cell migration, cell communication, and the cell clusters

will be investigated. Here the focus lies at the targeted differentiation of stem cells. Especially, the effect of vascularisation of differentiated stem cells *in ovo* will be investigated with deep interest. The advantageous immune-incompetent situation of the chicken embryo is especially beneficial in this regard as it prevents immune rejection of trans-species implantation/injection of cells and tissue constructs. The CAM of the developing embryo is highly vascularised and shows the potential for studying the *in ovo* vascularisation for tissue engineering. In parallel, the system is technically modified: open as far as possible, so that observation is possible in high resolution, without affecting the embryo. At last, systematically an artificial egg will be developed, where the requirement of different imaging methods, manipulation system, as also *in vitro* culture technique are suitable for the tissue engineering. Essentially, the system may not contain the geometry and the materials of a natural egg, rather will be replaced with artificial biomaterial in conjunction with fluidics.

The advantageous situation of *in ovo* culture will be imitated in the liquid | liquid interface culture system

- Free migration of cells,
- Application mechanical stimulation like the egg turning by the bird,
- Flexible, permeable substrates allowing diffusion,
- Better access to the cells for manipulation (addition/removal),
- Huge flexibility to add extra substrates,
- Easy removal of cells without enzymatic treatment (trypsin free passage),
- Good access for sensors, imaging and other technical modules.

Based on these features, a series of key-experiments will be conducted to test the feasibility of an alternative physiological *in vitro* culture approach for stem cells with the imitation of the embryonic development in avian egg. For this purpose, it is necessary to search for an appropriate stem cell model and to create the technological boundary conditions for reproducible experiments under microscopic documentation.

For the new bioreactor, it is necessary to change different natural egg component like shell, egg white and yolk egg membrane, many alternatives will be tested. Materials like porous Teflon, polycarbonate and silicone will not only be used, but also innovative appendages like hydrogel, and other high viscous or visco-elastic gel and fluids will be

investigated too. As substrate material perfluorochemicals, silicone and other material will be investigated. Following the key experiments, the components are integrated for use as a system for *ex ovo* culture. This system should be used together with the previous integrated imaging for the utilization of the final experiments.

At the end, characterised cell model should be implemented in the incubator and the *in ovo* situation will also be evaluated there for the establishment of a new *in vitro* liquid│liquid interface culture. Here the interest lies at the targeted differentiation of stem cells and neo-vascularisation of differentiated stem cells *in ovo*.

2.1 Goal

Goal of this thesis is the construction of a **"liquid│liquid interface culture system"** based on the guideline of the embryonic development in a fertilized chicken egg, which is intended for *in vitro* culture of mammalian cells and tissue engineering. Therefore, there are the following objectives

- Non-invasive very high resolution imaging of avian embryogenesis at different stages of development in closed eggs
- Addition of contrast labelled stem cells into the fertilized avian egg and traccking non-invasively
- Cultivation of avian embryos in open systems, bringing them to hatch
- Cultivation of avian embryo in completely artificial systems
- Step by step modification of the open system of avian culture and optimization for optical imaging
- Step by step technical modification of the open system of avian culture for placement of biosensors to access the bio-signals and installation of fluidics for addition and removal of culture medium
- Installation of micromanipulation system for addition and removal of cells
- Construction of a fluorescent micro imaging system for *in ovo* application enabling to the observation of cellular process inside the open system of avian culture.

2.2 Working plan

Within the framework of the thesis, liquid│liquid interface culture system should be developed and tested.

- At first, the avian embryogenesis will be observed with a very high resolution noninvasively in closed egg. This is necessary to follow the vital process of embryogenesis in its natural inhabitant. For the investigation, the fertilized quail egg will be incubated and imaged with Microscopic Magnetic Resonance Imaging (μMRI) in a very high resolution at different stages of development.

- During early embryogenesis, a single oocyte divides and an organized spatial distribution of cells gives rise to three different germ layers eventually forms a complete organism through the process of morphogenesis and organogenesis. The freedom of cellular movement and cellular migration will be studied non-invasively in its natural inhabitant. Stem cells will be labelled with contrast agent and will be injected into a fertilized egg through a small hole on the shell into or near the blastoderm. Their fate will be followed with μMRI non-invasively.

- To reveal the vital process of embryogenesis and to have better access to the embryo, the avian embryo will be cultivated in open system and in a completly artificial system. The open culture system will consist of surrogate shell taken from larger eggs (recipient), which will contain the avian embryo and covered with plastic film. The completly artificial system will consist of porous Teflon membrane suspended on stainless steel mash and covered with plastic film. This will start the gradual modification of natural avian egg towards more artificial system.

- Gradual technical modification of the open system of avian culture. This technical modification is necessary, since avian eggs are not suitable for high-resolution optical imaging, and for ultrasound and other possible imaging and measurement methods (hard calcareous shell is non-penetrable for ultrasound, opaque, not feasible for micromanipulation and there are sterility problem). This technical innovative approach will open a new horizon. In the process of technical modification, part of the shell will be gradually replaced by biocompatible transparent window material; the covering lid will be optimized for optical microscopy and easy access for

micromanipulation system for the addition and removal of cells. Besides application of different biosensors will be carried out in order to access bio-signals. This may include thermal sensors near the embryo, flexible electrode to record bio-electric signal and impedance measurement, p^H measurement and gas sensors like O_2 and CO_2. Application of fluidics is another very important issue. Since the whole process is intended for *in vitro* cell culture, addition and removal of culture medium is necessary for oxygenation, removal of CO_2, addition of nutrients and growth factors. For future modifications, biochip based sensors can be implemented for biochemical and immunoassay which will reveal the secret processes of cellular communication, cell signalling, organised cell migration in groups, morphogenesis, differentiation, tissue formation tissue, organogenesis and the complete process of embryogenesis. This may open a completely new chapter in the history of cell culture and tissue engineering. As described in chapter 1, the current process of *in vitro* cell culture and tissue engineering is stuck at a certain point. The researchers are trying different ways to overcome the obstacle but there is not much progress. Actually, the non-physiological way practiced *in vitro* is getting away from the *in vivo* situation.

3 µMRI of avian embryogenesis *in ovo*

3.1 Summary

Objective: Important procedures in tissue engineering include the isolation and *ex vivo* proliferation organotypic cells and the seeding of these cells into suitable biocompatible matrices. However, the limiting factor for the survival, proliferation, and differentiation of transplanted cells is the sufficient supply of nutrients and oxygen, which relies entirely on diffusion processes. *In ovo,* this is achieved by angiogenesis, which is not possible *in vitro* until now. During embryogenesis, single fertilized oocyte gives rise to a multicellular organism. In the avian embryo, the extensive capillary network of CAM achieves this transport for the developing embryo. To understand tissue generation *in ovo* requires very high resolution imaging that allows imaging at cellular level without disturbing the normal embryonic growth and development. For *in ovo* imaging of the avian embryogenesis, the calcareous shell is the main obstacle for optical imaging methods. A thin layer of gas between eggshell and shell membrane along with calcareous shell make ultrasound inapplicable. Computed Tomography (CT) and Positron Emission Tomography (PET) could be applicable in this aspect but they rely on ionising radiation for imaging contrast, which may load the embryo with the invasive dose of ionising radiation. Therefore, the option available is Magnetic Resonance Imaging Microscopy (µMRI).

The main purpose of the experiments in this chapter was to make highest resolution (as high as 39 µm × 39 µm) µMRI imaging of quail (*Coturnix coturnix*) embryos within reasonable time. Quail embryogenesis was studied with µMRI imaging *in ovo* keeping the animal alive. Contrast labelled stem cells were injected into quail embryos and they were tracked with µMRI. MR terminology follows the common convention that microscopic resolution means the ability to distinguish structures equal to or smaller than the optical resolution of the human eye (i.e. about 100 µm).

Methods: A combination of very high magnetic field strength (B_0 = 9.4 T), a high sensitivity probe and strong magnetic field gradients was employed to record MR images from quail (*Coturnix coturnix*) embryos at different stage of development. Rat

pancreatic stem cells (Z29 P24) loaded with Super Paramagnetic Iron Oxide Nanoparticle (SPION), injected into fertilized quail eggs and tracked them with µMRI.

Results: The *in vivo* microscopic MR imaging series revealed the process of embryonic development in different stages. All these processes have been observed in detail before but the non-invasive approach taken here allows monitoring the entire embryological development of an individual avian embryo inside an opaque eggshell. The opaque eggshell represents a major obstacle for optical methods. Furthermore, cellular labelling of stem cells with SPION allowed tracking of cellular migration *in ovo*.

Conclusions: MRI is capable of revealing the process of embryonic development at very high resolution. Besides cell tracking opportunities make it a versatile tool for developmental biology and stem cell research. The imaging acquisition techniques of MRI are relatively time consuming. Nevertheless it matches with the slow process of embryonic development. The embryo begins to move physically in the course of development; especially the heart starts to beat very early. This brings motion artifacts into the image and makes it more complicated for MRI imaging. Besides construction of an incubator inside the imaging magnet was difficult to achieve for *in ovo* imaging of quail embryos (optimization of temperature, humidity, rocking of eggs and more). At the end it was concluded that µMRI is not a method to study embryogenesis *in ovo*.

3.2 Materials required for µMRI experiments

Equipments	Manufacturer
Centrifuge, 3K18	Sigma-Aldrich, Deisenhofen
DMX 400 spectrometer with micro 2.5 gradient system	Bruker BioSpin, Rheinstetten
Lumix FZ50 digital camera	Panasonic, Japan
Microscope	Olympus, Hamburg
Magnetic separators MagnetoPURE magnetic plate	chemicell GmbH, Berlin
Refrigerator (4 °C)	Liebherr, Biberach a. d. Riss
1T/m gradient system	Bruker BioSpin, Rheinstetten
9.4 T superconducting vertical bore magnet	Do
25 mm Bird cage micro imaging coil	Do
176/M2 Motorbrüter with semiautomatic rocking	Bruja, Hammelburg

Chemicals	Manufacturer
Agarose	Peqlab, Erlangen
Bacillol anti-microbial solution	Bode Chemie, Hamburg
FluidMAG-CMX/E (Carboxymethyl-Dextran)	chemicell GmbH, Berlin
Hypochloride solution (200-500 ppm)	VWR International GmbH
PBS	Invitrogen, Karlsruhe

Biological agents	Manufacturer/supplier
Fertilised quail eggs	wachtelei-spezialist, Aletshausen
Rat pancreatic stem cells (Z29)	Fraunhofer IBMT, Lübek

Computer Software	Developer
Amira®	ZIP, Berlin
ImageJ	NIH, USA

Accessories	Manufacturer/supplier
Glassware	Schott, Mainz
Microinjection syringe	Eppendorf, Hamburg

3.3 Considerations for *in ovo* imaging

Before going into the detailed discussion of *in ovo* imaging, the structure of the egg shell needs to be explored. Avian egg is covered with a hard, protective shell which is opaque. An average egg of 60 g has 343 µm shell thickness has about 12000 pores per egg. Average pore length is 300 µm with an average pore diameter of about 3 µm [340]. The true crystalline shell is composed primarily of calcium carbonate in the form of calcite with just a trace of aragonite and octacalcium phosphate. The shell is associated with double layer shell membranes on the inside and the cuticle (or cover) on the outside of the egg. The shell membranes are consisted with a network of fibers several layers thick. The two membranes adhere to one another to form a compound membrane; except at the blunt pole of the egg where they separate to form the air space. Fibers of the inner shell membrane are finer and form a tighter mesh than those of the outer membrane. The shell along with its membrane is structurally & architecturally a stable structure which is perfectly designed for protecting the developing birds even from bacterial invasions.

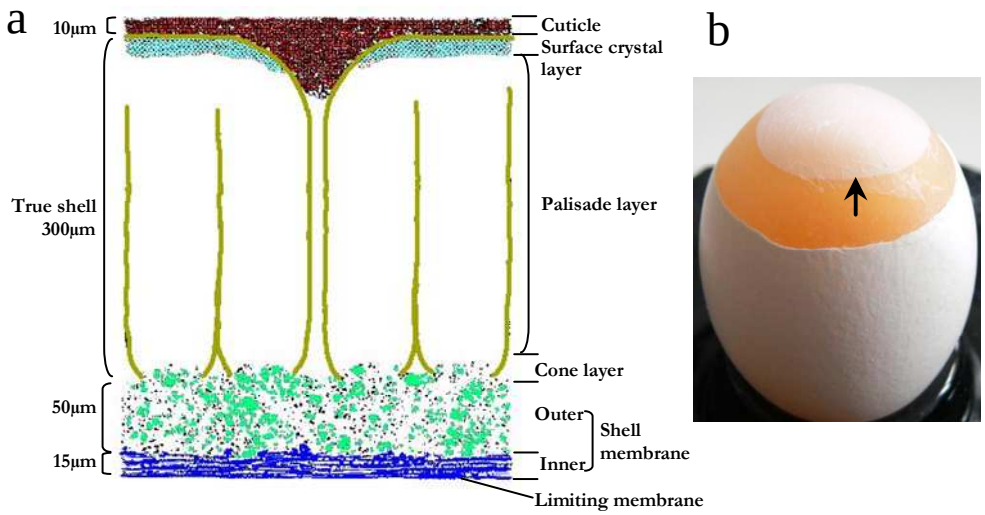

Figure 3.1: Structure of chicken eggshell.

(a) Radial section through a domestic fowl eggshell showing the main structural features. Numbers on the left of the diagram are thicknesses of each layer in µm (redrawn after Tullett, S. G., 1984 [352]). (b) Eggshell removed from the blunt pole of a chicken egg showing the air cell trapped between two layers of shell membrane (arrow indicates the air cell).

The *in ovo* imaging has to be performed through the hard, opaque eggshell keeping the embryo alive. There are different imaging methods offering non-invasive

imaging possibilities with their own advantages and disadvantages. These include Ultrasound, CT, PET, µMRI, optical methods & others.

Ultrasound

Ultrasound relies on the modification of an induced acoustic wave travelling through tissue. Ultrasound studies are conducted using a probe to project sound into the tissue and recording the time and magnitude of the reflected sound wave using the same probe. The analysis of this acoustic echo permits the imaging and measurement of tissue acoustic properties. It is used primarily for monitoring tissue structure and motion. The resolution of ultrasound is roughly proportional to the wavelength of operation. However, the ultrasound is reflected al the interface of changing material density or the large change of the sound speed. The egg contents are enclosed in a hard, calcareous, bonelike eggshell and there is a thin film of air between the calcareous eggshell and shell membrane.

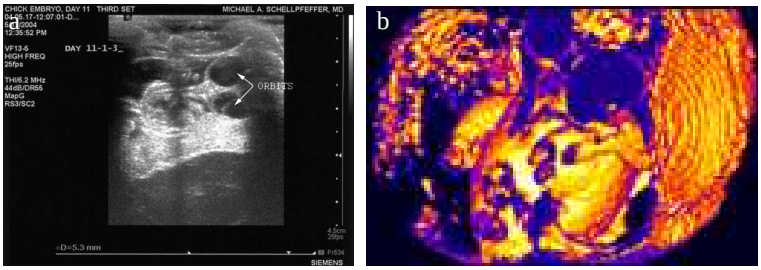

Figure 3.2: Comparison between Ultrasound and µMRI image.

(a) Ultrasound image of chickcken embryo at ID 11 [295], (b) 3D surface reconstruction of MRI T_2 weighted image of a quail embryo inside the egg at ID 12.

In spite of the advantages of ultrasound including the ease of use, portability, and relatively low cost compared with MRI, CT, and PET; the amount of tissue characterization that can be accomplished with ultrasound is limited. Because of hard eggshell and a thin air film in between the calcareous eggshell and shell membrane, it is not applicable in the avian eggs.

Computer tomography (CT)

CT is basically a three-dimensional x-ray technique that is sensitive to the x-ray absorption of the tissue. Contrast can be generated by the differences in tissue absorption, with bone providing the most striking intrinsic contrast, or by using contrast

agents to enhance the vasculature or specific tissues and conditions. The inherent Signal-to-noise ratio (SNR) of CT is very high.

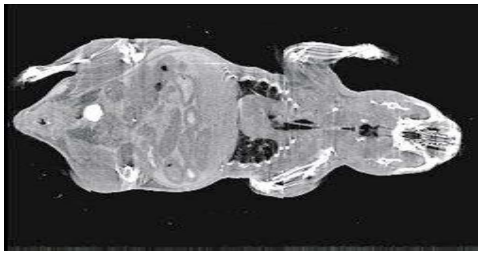

Figure 3.3: Computed tomography coronal section through a whole mouse.
(Image courtesy of Oak Ridge National Laboratories, Oak Ridge, Tennessee).

Using current technology, full three-dimensional mouse images with 100 µm x 100 µm x 100 µm resolution can be obtained in a few minutes [107, 154, 252, 397]. With higher resolution studies approaching potentially 50-µm isotropic resolution, with the one limitation being that the amount of energy absorbed by the animal may approach "invasive" levels. In case of *in ovo* imaging, eggshell remains a problem. High radiation absorption by the calcareous shell results in high exposure or high-energy imaging. This will worsen soft tissue contrast. Besides, high radiation dose and use of ionizing radiation makes CT iapplicable for *in ovo* imaging.

Positron Emission Tomography (PET)

PET relies on detection of radioactive probes emitted in the body. Imaging of this emission is performed using a combination of detector geometry along with the timing of the emissions detection. PET is one of the most sensitive imaging techniques and is capable of detecting vanishing small amounts of radiolabelled material. The short-lived isotopes [139] used in this approach include ^{11}C [171, 185], ^{13}N, ^{15}O [192, 396] and ^{18}F [139, 396] isotopes, which are extremely useful in the evaluation of biological processes. The sensitivity of PET has resulted in a unique ability to monitor receptor ligand interactions in humans and animals with remarkable success. This sensitivity has resulted in PET being one of the primary targets in the development of gene expression markers as well as the detection of early cancer [258]. One of the major drawbacks of PET is the requirement for a local cyclotron to generate the probes and a synthesis unit to produce the biologically useful probes. Because radioisotopes must be used in these studies, vascular access or direct injection of the tracers into the organ of interest is required.

Laser scanning microscopy

Laser scanning microscopy has continued to develop and has proved itself to be an excellent tool for *in vivo* microscopy due to its ability to collect 'optical sections' through the specimen. By raster scanning a laser beam over the focal plane and blocking light from elsewhere with a confocal pinhole in front of the detector, an image largely devoid of out-of-focus fluorescence can be generated. Confocal Laser Scanning Microscopy (CLSM) works well for imaging near the surface (within 100 µm). At greater depths, scattering in the tissue and blurring due to the tissue optics make collection of light through a detector pinhole inefficient. A more significant limitation is the photo bleaching from CLSM, because it excites fluorescence throughout the depth of the specimen even when it is collecting a single optical section. Despite these difficulties, CLSM images have offered important glimpses into the developing embryo [279, 374]. In preparations with no absorbance of the intense infrared pulses and limited light scattering, two-photon microscopy has permitted observation of live cells and intact tissues with startling resolution [238, 262]. Because of hard, calcareous eggshell, Laser scanning microscopy is not applicable for *in ovo* imaging.

Therefore, in case of *in ovo* imaging of avian eggs, only µMRI seems to be applicable.

3.4 Microscopic Magnetic Resonance Imaging (µMRI)

In short, magnetic resonance is the absorption of electromagnetic energy by a subpopulation of atomic nuclei in an external static homogenous magnetic field when irradiated at an isotope specific resonance frequency directly proportional to the local magnetic field strength. When the absorbed energy is released upon return to the thermal equilibrium, an inductive signal can be observed which contains chemical and – under special conditions – spatial information about the molecular composition of the irradiated sample. The concept has repeatedly found comprehensive treatment elsewhere – (see, e.g., [2, 306] for spectroscopy and [29, 45] for imaging) and will therefore only briefly be sketched here.

Atoms exposed to an external magnetic field B_0 experience a Zeeman splitting of their energy levels such that the magnetic quantum number m can take on all integer

values between +*I* and –*I*, the extremal values of the spin quantum number *I*, provided that $I \neq 0$. Atoms with even numbers of both protons do not fulfill the latter condition and neutrons because the individual spins of identical nucleons cancel out. For the nucleus most commonly employed in MR studies and in this thesis, ^{1}H, $I = 1/2$.

The Zeeman energy difference $\Delta E = Ei - Ej$ between adjacent ($\Delta m = 1$) energy levels Ei and Ej (with $Ei < Ej$) can then be expressed as

$$\Delta E = \frac{h}{2\pi}\gamma B_0, \qquad \text{Equation 3.1}$$

where γ is the gyromagnetic constant of the isotope ($2.67522 \cdot 10^8$ s^{-1} T^{-1} for ^{1}H), and $h = 6.62607 \cdot 10^{-34}$ J is the Planck constant.

The equilibrium populations of both energy states follow a Maxwell-Boltzmann distribution:

$$N_i / N_j = e^{-\Delta E / kT} \qquad \text{Equation 3.2}$$

with Ni, j representing the number of nuclear spins in state i and j with the respective energy level, $k = 1.38065 \cdot 10^{-23}$ J/K the Boltzmann constant and T the temperature. For ^{1}H in a magnetic field of 9.4 T and a temperature of 300 K, this translates into a population difference $\Delta N = N_i - N_j$ of about sixty spins out of one million, which severely restricts the sensitivity of the method.

As a consequence of the population difference, a net magnetisation M_0 can be observed along the axis (usually named z) of the static field B_0. The equilibrium distribution can be disturbed by supplying $\Delta E = h\nu_0$ via radiofrequency (rf) pulses oscillating at a frequency ν_0 around an axis perpendicular to z, which creates an additional magnetic field B_1. With Equation (1.1), the resonance condition for the absorption of this pulse is given by the Larmor relation

$$\omega_0 = 2\pi\nu_0 = -\gamma B_0, \qquad \text{Equation 3.3}$$

Between the angular frequency ω_0 of the rf pulse and the local magnetic field (for the experiments considered in this chapter, B_0 = 400 MHz at 9.4 T). Such a rf pulse will stimulate transitions from the lower to the higher energy state (as long as $\Delta N > 0$), thereby flipping the net magnetisation in the sample by the flip angle

$$\theta = \gamma B_1 t_{flip} \qquad \text{Equation 3.4}$$

where t_{flip} is pulse duration time

When the spins relax by releasing the absorbed energy, a so-called free induction decay (or FID) induces a signal *S* in a receiver coil. For the typical case of a spin echo pulse sequence (two pulses corresponding to flip angles of $\pi/2$ and π, separated by an echo time TE), the generated signal *S* (*t*) is of the form:

$$S(t) = C\rho(1 - e^{TR/T1})e^{-TE/T2}, \qquad \text{Equation 3.5}$$

where *C* is a constant for a given sample, while ρ represents the spin density in the sample, T_R the repetition time (i.e. the interval between the onset of two consecutive rf pulse sequences), T_1 the longitudinal relaxation time (i.e. the time constant of the magnetisation decay longitudinal to the static magnetic field, also known as spin-lattice relaxation time), T_E the echo time (i.e. the time between the onset of rf pulsing and the beginning of signal acquisition) and T_2 the transverse relaxation time (i.e. the time constant of the magnetisation decay perpendicular to the static magnetic field, also known as spin-spin relaxation time). It should be noted that the refocusing function of the second pulse at $\theta = \pi$ can alternatively be fulfilled by the administration of appropriate field gradients. Such schemes are known as gradient-echo sequences which show the same signal decay as described by Equation 1.5, except that T_2 has to be replaced by the apparent relaxation time T_2* which is shorter than T_2, as the loss of transverse magnetisation in gradient-echo sequences arises not only from the microscopic spin-spin interactions causing T_2 decay but also from macroscopic magnetic field inhomogeneities.

Fourier transformation of the time domain signal described by Equation. 1.5 generates a spectrum in the frequency domain where the position, form and ratio of the peaks give information about the molecular composition of the sample in terms of the employed isotope. This is the basis of MR Spectroscopy (MRS).

Typically, at least in the life sciences, MRS is performed in aqueous samples but methods have also been developed to investigate solids. Such a variant is called Magic Angle Spinning (MAS) because the sample is quickly spun around an axis inclined to the

static magnetic field by the "magic angle" (about 54.7°) where spatial magnetic dipolar interactions between spin pairs are effectively averaged out [14].

A further implication of Equation. 3.3 is that the magnetic field can be modulated in time and space by manipulating radiofrequency pulses such that they create space-dependent magnetic field gradients $\vec{G}(\vec{r})$ supplementary to the static magnetic field:

$$\vec{G}(\vec{r}) = \frac{\partial B_z}{\partial x}, \frac{\partial B_z}{\partial y}, \frac{\partial B_z}{\partial z}, \qquad \text{Equation 3.6}$$

In practice, this is achieved by employing a dedicated spatial arrangement of gradient coils that send gradient pulses each time an rf pulse sequence is applied. If the time that the gradient remains on is varied between consecutive rf pulses but its amplitude is kept constant, this regime is referred to as frequency encoding. Conversely, phase encoding depicts the concept of varying the gradient amplitudes and keeping them on for a constant period.

The increase of SNR with increasing B_0 is the main rationale behind the quest for ever-increasing field strengths of MR magnets, especially for spectroscopic applications and medical imaging [50]. Nonetheless, it should be stressed that the multiparametric nature of MR also brings about negative effects of higher field strengths (e.g. eddy currents) or positive effects at lower field strength (e.g. line narrowing) on the quality of MR data [65, 109] and that even NMR at Earth's magnetic field strength is possible with a vary low sensitivity [46] [the strength of the magnetic field at the Earth's surface ranges from less than 30 microteslas (0.3 gauss) in an area including most of South America and South Africa to over 60 microteslas (0.6 gauss) around the magnetic poles in northern Canada and south of Australia, and in part of Siberia].

Small animals permit the use of small magnetic resonance receiving coils, which increase the sensitivity to the magnetic fields generated by the nuclides. In other words, the closer a coil can be physically placed to a target organ, the better the SNR of the measurement (so called filling factor). The SNR of the MRI experiment roughly increases linearly with the magnetic field when the sample noise dominates. At the time of this writing, mouse studies can be conducted on 11.7 T or even higher field systems in comparison to the 1.5-T systems used in humans [T = Tesla or 10,000 gauss; earth's field is 30 microteslas (0.3 gauss) to 60 microteslas (0.6 gauss)]. This means that an

approximate factor of 10 or more increase in SNR can be realized using these magnets. For example, if an imaging voxel is 2 mm × 2 mm × 20 mm at 1.5 T, the voxel can only be reduced isotropically to ≈ 0.9 mm × 0.9 mm × 9 mm at 11.7. Thus, both the magnetic field and coil proximity issues must be used to optimize the MRI experiment on a small mammal [image optimization using Field of View (FOV), pulse sequences, gradient strength, and temperature optimization].

Magnetic resonance imaging microscopy (µMRI) is a qualitatively different imaging method that is able to distinguish soft tissues within optically opaque specimens. During a µMRI experiment, 3D data are collected directly from the intact specimen; thus, no artefacts are introduced by the physical deconstruction of the specimen. This non-invasive method offers several different sources of contrast and makes it possible to repeatedly image the same specimen over time [142]

Contrast agents in ^{1}H MRI are usually metal-based agents (including free Mn) that modify the magnetic relaxation properties of water. This permits the elimination or enhancement of the water signals depending on the agent and detection scheme. Specifically, agents to enhance the vascular bed or distribute in the interstitial space have been very useful in angiography [30, 179] as well as perfusion [137, 189], tumour detection [202, 289], and neuronal fibre tracking [253]. MRI and MRS methods have been successfully applied to the mouse and rat due to the advantageous scaling factors that occur in magnetic resonance. Microscopic magnetic resonance imaging (µMRI) offers a powerful imaging modality for structures that absorb or scatter too much light. When compared to optical methods, MRI is low resolution, but it has the ability to generate startling images in even the most difficult preparations. Contrast in the MRI images results from differences in the magnetic properties and/or local environment of the water protons contained in a specimen. The exact nature of the image contrast can be adjusted by altering excitation (pulse) and timing (echo) parameters used for image acquisition. The radio frequency radiation is not directly imaged; therefore, MRI is not limited to optical properties of the specimen. Beyond being able to ‘see’ deep within opaque specimens, MRI can reveal features not easily detectable in optical images. By imaging the diffusion tensors, regions that restrict or facilitate water diffusion can be visualized [217]. One huge advantage of MRI is that the object being imaged is kept intact; the data remains inherently registered allowing for rapid 3D reconstruction (e.g. a rare 3D imaging

sequence requires 1 h and 8 min for an image with 128 x 128 x 128 image points with a repetition time of 2000 ms und 4 accumulations, with 8 accumulations double the time, 2 h 16 min)

Unpaired protons placed in a strong magnetic field precess at a very explicit frequency. These protons interact with an applied oscillating magnetic field at the same (resonant) frequency, absorbing and re-emitting energy in ways that can be spatially encoded to generate a digital image. The detection of the naturally occurring nuclide ^{1}H found in water and fats is usually used for MRI studies providing an adequate SNR to create images with sub-millimetre resolution *in vivo*. Magnetic resonance microscopy (MRM / µMRI) is based on the same physical principles as its clinical cousin, MRI.

For the aim of the Ph.D. work, the following aspects are interesting for high-resolution imaging of the avian embryo:

- µMRI is non-destructive
- It takes advantage of unique "proton" stains
- It is inherently 3-dimensional
- It is inherently digital.
- The specimen can be sliced along any desired plane
- The specimen can be scanned in many different ways, at different resolutions, through different planes.
- The two slices can be carefully matched to compare the anatomy. Since no distortion occurs from dehydration and physical sectioning, morphometric measurements are much more accurate than could ever be made with traditional glass slides.

3.5 Experimental approaches

µMRI allows *in ovo* imaging of avian embryo inside eggshell non-invasively. In this chapter fertilized eggs of common quail (*Coturnix coturnix*) is imaged *in ovo* at different stage of development. Incubation time of the quail is 17 days, the average egg weight is ≈15 g, and diameter (short axis) of the egg is ≈25 mm.

3.5.1 Preparation of the embryos

Fertilized quail eggs were purchased and transported from local farms. On arrival, eggs were washed with Chlorine solution (Annexes, egg-cleaning protocol) and

preserved in a refrigerator at 14 °C for 1 day. Eggs were than incubated in a forced air incubator (Bruja Motorbrüter, Model 176/M2 with semiautomatic rocking) at 37.5 °C, 65% relative humidity and 90° rocking from side to side in an hourly cycle. Embryos were prepared for imaging at different stage of incubation. To avoid movement artefacts during switching of the gradient coil at the time of imaging, eggs were wrapped with Teflon foil and placed tightly inside the µMRI-imaging coil (Bird cage, 25 mm inner diameter from Bruker BioSpin, Germany). The imaging coil with the embryo containing eggs was loaded into the superconducting magnet for imaging.

3.5.2 Parameters for the high resolution µMRI experiments

3.5.2.1 Imaging

Fertilized quail eggs were imaged inside the eggshell with Bruker DMX 400 spectrometer with micro 2.5 gradient system and 9.4 T superconducting vertical bore magnet (He/N_2 cooled) from Bruker BioSpin equipped with 1 T/m gradient system. Fertilized quail eggs were incubated in a forced air incubator. Incubated quail eggs at different embryonic stage were placed inside the imaging coil and the coil is inserted in the magnet. Images were acquired using spin-echo sequences (MSME routine, Bruker Biospin) inversion recovery and rare sequences (Order rare, Bruker Biospin). Aim of the imaging was to get maximum contrast between the developing embryo and egg contents. Image processing was performed using ImageJ [138] which is a Java-based image processing program developed at the National Institutes of Health and Amira® 4.1.0 image processing software from Mercury Computer Systems.

3.5.2.2 Magnetic Resonance Spectroscopy (MRS)

Magnetic resonance Spectroscopy (MRS) generally gathers the spectral information in the magnetic signals from the nuclides, which permits the determination of the molecules or metabolites containing a given nuclide. The collection of this additional information in MRS along with the fact that metabolites are generally at low concentration results in the MRS experiment having a low SNR. These combined effects make any images collected with MRS very poor in spatial and temporal resolution. MRI and MRS must be conducted in a strong homogeneous magnetic field, which requires a specialized magnet as well as receiver coils to detect the nuclide signals. Because the

absorption of these oscillating magnetic fields is relatively low in biological tissues, the penetration of these signals is excellent in most studies. The detection of the naturally occurring nuclide ^{1}H found in water and fats is usually used for MRI studies providing an adequate SNR to create images with sub millimeter resolution *in vivo*. The MRI signal from water protons is rich in informationn about the physiology and function of tissues because it is the solvent of the cell with very little occurring without some impact on the magnetic properties of this molecule [23]. This information includes a diverse amount of information on blood flow and oxygenation as well as macromolecular composition and motion, tissue structure, temperature, contractile activity, nerve and muscle fibre orientation, and oedema.

The aim of the Magnetic Resonance Spectroscopy (MRS) was to differentiate between the two different types of egg white: the thin and the thick. ^{1}H and ^{13}C spectroscopy were performed with the loaded sample. To improve the resolution, high resolution spectroscopy was carried out (Magic angle spinning).

Figure 3.4: µMRI imaging system.

(a) 9.4 Tesla superconducting vertical bore magnet from Bruker BioSpin, Rheinstetten, Germany. (b) 25 mm Bird cage micro imaging coil containing a quail egg (scale bar = 10mm)

3.5.2.3 Magic Angle Spinning (MAS)

In MAS, thin and thick egg white was loaded in a tiny ceramic tube (Ø = 4 mm, 14 mm long) leaving no air inside & the tube containing the sample is rotated at a very high rotation speed (up to 20,000 Hz) in homogenous and high magnetic field and

spectroscopy was performed. This method gives higher resolution to differentiate fine chemical shifts between samples. For both types of egg white, two separate experiments were carried out. The data was analysed with Origin8 data analysing and graphing software. It is important to notice that MAS was not performed with the whole egg.

3.5.3 Injection of contrast labelled stem cells into the fertilized quail eggs and tracking of them *in ovo*

Aim of this experiment was to inject contrast labelled stem cells into fertilized quail eggs near the embryo and track them noninvasively. Rat pancreatic stem cells (Z29 P24) were labelled with Super Paramagnetic Iron Oxide Nanoparticle (SPION) FluidMAG-CMX/E (Carboxymethyl-Dextran) (chemicell GmbH, Berlin, Germany) and labelled cells were injected into a fertilized quail egg through a small hole at the blunt end. The whole egg was imaged with MRI to track the cellular fate. The SPION concentration was 25 mg/ml and particle size was 50 nm.

Among the large family of magnetic materials, ferro- or ferri-magnetic materials are found in massive materials or submicroscopic particles: the magnetic susceptibility is large, the magnetization increases massively with the magnetic field intensity, a remnant magnetization occurs after removal of the magnetic field and, above a critical temperature called the Curie temperature (Tc), the ferro or ferri-magnetic behaviour disappears. The largest iron oxide nanoparticles belong to this class (e.g., Tc = 580 °C for Fe_3O_4). If their size is below approx 10-20 nm, iron oxide nanoparticles are said to be "superparamagnetic" and their main feature is the absence of remnant magnetization, limiting magnetic interactions within particle and, therefore, facilitating their stabilization as ferrofluids in aqueous solutions.

SPIONs are composed of magnetite ($FeOHFe_2O_3$). These particles have magnetic moments that, because of the small crystal size, are unhindered by lattice orientation and therefore do not exhibit hysteresis, hence the term "superparamagnetic." In an applied magnetic field, the individual moments are free to align along the field, resulting in the formation of a single spin, with a net moment at least four orders of magnitude higher than a comparable ensemble of paramagnetic spins. This creates extremely large microscopic field gradients for dephasing nearby protons [38, 41]. This, in turn, dramatically shortens the nuclear magnetic resonance T_2 relaxation time, over and far beyond the

usual dipole–dipole relaxation mechanism that affects both T_1 and T_2, as in the case of paramagnetic contrast agents. Owing to the predominant T_2 effect, these "T_2 agents" usually create hypointense contrast on conventional spin-echo MR sequences, in particular when agglomerated within cells. Agglomeration or intracellular clustering of iron oxide nanoparticles dramatically reduces the T_1 effect [39]. Iron oxide particles are highly suitable for this purpose because they cause a strong local disruption of the homogeneity of the magnetic field and a loss in MR signal, which makes labelled cells appear black.

Even though iron is an essential component of many enzymes and proteins, it can be toxic in high concentrations [54, 60]. In some cells, such as liver macrophages, relatively small increases in intracellular iron can have deleterious effects on cellular signalling and function [183, 380]. Iron accumulation in tissues also catalyzes the Fenton reaction and potentiates oxygen toxicity by the generation of a wide range of free radical species, including hydroxyl radicals, which are the most active free radicals known and have the ability to react with a wide range of intracellular constituents [264, 381] and may lead to cell death [83, 111]. For stabilization in order to prevent aggregation & to make them biocompatible, they are coated with macromolecules like dextran.

However, the dextran surface of the nanoparticle is unfavourable for inducing endocytosis and for effective MRI visualization of cells in vivo, the concentration of iron intracellularly has to be much higher (≈10 pg per cell). This concentration exceeds physiological concentrations ≈100-fold. Cellular uptake of dextran coated nanoparticles vary from 0:011 to 0.118 pg of iron per cells in different tumour cells and a maximum load of 0.97 pg in primary isolated peritoneal mouse macrophages [215, 216, 299], which is much lower than the imaging threshold. Incubation of cells with SPION on a magnetic plate is claimed to improve magnetic load. However, labelling efficiency was not improved much.

Most current labelling techniques use one of two approaches: extracellular labelling by attaching iron particles to the cell surface [114] or internalizing magnetic markers inside the cell [39, 188, 297, 398]. For intracellular uptake of iron particles, several different techniques are used: fluid phase endocytosis [188, 297], conjugated antibodies [398], and magnetodendrimers [39]. These methods all have their disadvantages. After extracellular labelling, either the label can be easily lost or it can interfere with cells to-

cell interactions. The intracellular methods are either relatively ineffective (fluid phase endocytosis), require specialized laboratory techniques (magnetodendrimers), or are cell-type specific (conjugated antibodies).

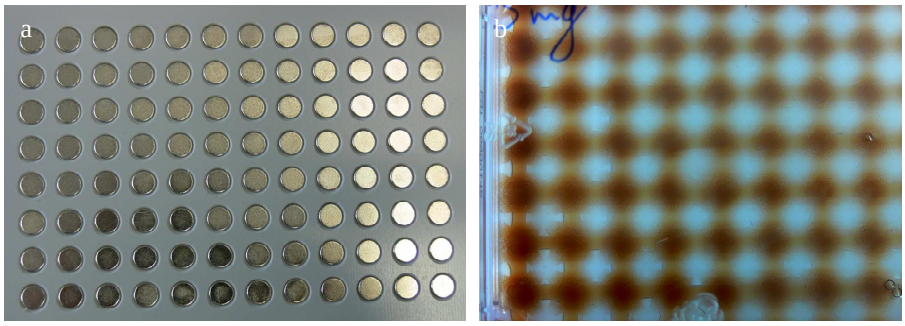

Figure 3.5: Magnetic separators.

(a) Magnetic separators MagnetoPURE magnetic plate (ChemiCell GmbH, Berlin, Germany), (b) Rat pancreatic stem cells (Z29 P17) incubated with FluidMAG-CMX/E with 30 ml culture medium in T175 flask of confluent cells (5-7e6 cells) (ChemiCell GmbH, Berlin, Germany). In image (b), magnetic particles are concentrated over tiny magnets in the culture flask.

Lipofection

Simply incubation the cells with SPION don't load enough Iron into the cells to be used as contrast method. Since egg yolk has much lower T_2 weighted value (≈30 ms), the Iron content has to be much higher to be detectable with MRI.

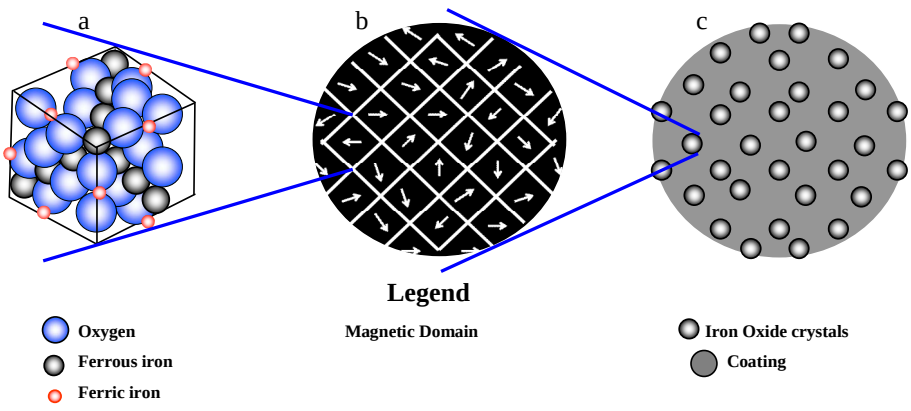

Figure 3.6: Schematic representation of a spinal crystal structure for superparamagnetic iron oxide Nanoparticle (SPION) domain.

(a) Crystalline arrangement (b) SPION crystal with multiple magnetic domains of random orientation and (c) complete SPIO contrast agent particle, with multiple SPION crystals and coating material. Redrawn after Wang et al, 2001 [377].

A new labelling technique based upon the use of cationic liposome transfection reagentsis free from thesedisadvantages [94]. By mixing the SPION formulation and the transfection agent, complexes of the two are formed through electrostatic interactions

[145]. When the complexes are added to the cell culture, the transfection agent effectively shuttles the SPION into the cell through formation of endosomes. Labelling can be improved (≈100-fold) with lipofection technique, generally used for the infusion of DNA into cell nuclei [363].

By using a short incubation time and low extracellular iron concentration, it is possible to achieve an efficient iron load without affecting cell viability & physiology. With a symmetric cell division and even dilution of magnetic label among progenitor cells, Individual magnetically labelled cell with an initial iron content of 25 pg can be detected up to at least four cell divisions [40].

Visualization of intracellular iron

To visualize intracellular iron content, Prussian Blue staining was performed (Annexes for details). A stain for ferric iron as in haemosiderins, using potassium ferrocyanide in acetic acid or dilute hydrochloric acid followed by a red counterstain such as safranin O or neutral red; various haemosiderins and most mineral irons give a blue-green reaction, while nuclei stain red. Perls' Prussian blue (Perls, 1867) solution was freshly prepared

3.6 Results

3.6.1 *In ovo* µMRI of the quail embryogenesis

3.6.1.1 µMRI image of the nondeveloped fertilized quail eggs

Figure 3.7 shows the µMRI image of a fertilized quail egg before incubation. In the first image (inversion recovery image) the latebra is clearly visible as a pear shaped bright structure inside the egg yolk [green arrow in image (a) and (b)].

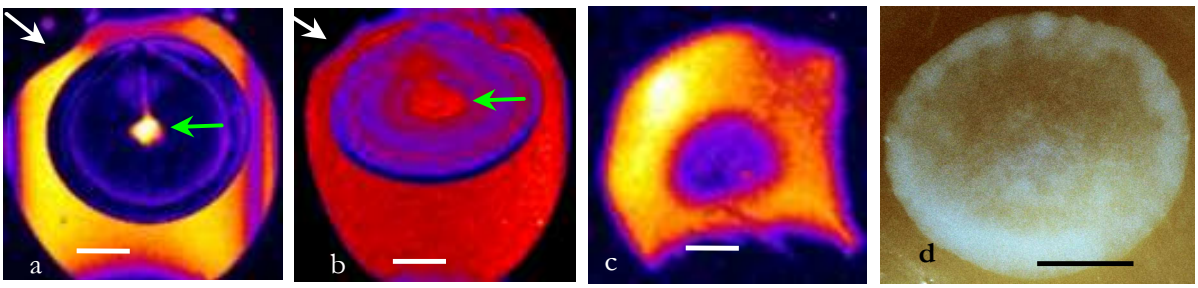

Figure 3.7: µMRI image of fertilized Quail egg.

(a-c) µMRI image of fertilized quail egg and blastoderm before incubation; (d) optical image of the blastoderm surface before incubation. (a, c) Inversion recovery image; (b) T_2 weighted image. (a-b) Longitudinal section through long axis cutting through the blastoderm, (c) zoom MRI image at the blastoderm surface. In the inversion recovery image of the whole egg (a), latebra, which is a part of white yolk, is clearly visible (green arrow). In T_2 weighted image (b), alternating layer of yellow and white yolk and latebra is clearly visible. White arrows indicate the air cell. (a-b) Slice thickness 1mm, pixel size = 195 µm × 235 µm; (c) slice thickness 0.5 mm, pixel size = 39 µm × 39 µm. Scale bar: (a-b) = 5 mm, (c, d) = 1mm.

The tail of the latebra ends at the sub-germinal cavity. This is actually a very good guide to find the position of the blastoderm. The egg yolk is deposited as concentric ring at day and night alternatively. The latebra is a part of white yolk which is deposited during the day. In T_2 weighted image, the concentric rings of yellow and white yolk are visible.

Figure 3.8 shows comparison of different parts of non-developed egg between MRI and schematic view.

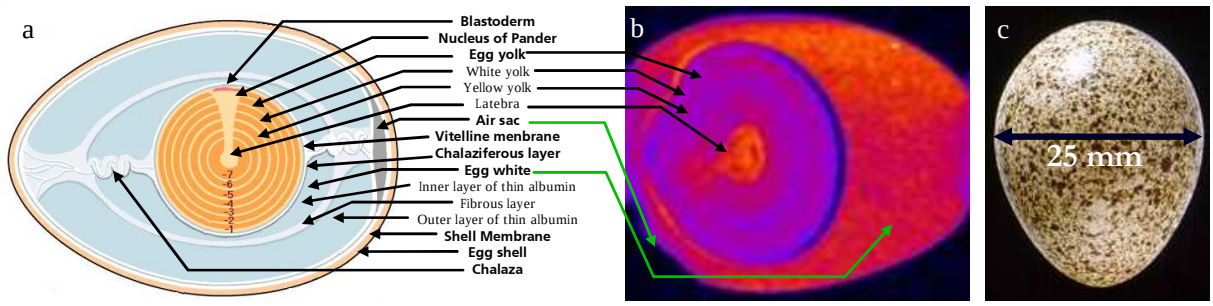

Figure 3.8: Different parts of avian egg

(a) Schematic view, (b) MRI T_2 weighted view, (c) Intact quail egg.

3.6.1.2 µMRI image of the quail embryogenesis - after 24 hrs incubation

Figure 3.9 shows the µMRI image of a quail embryo 24 hrs after incubation. The developing embryo is clearly visible as a bright spot in both T_1 (image a) and T_2 weighted image (image b). Latebra is marked with green arrow in image (a) and (b).

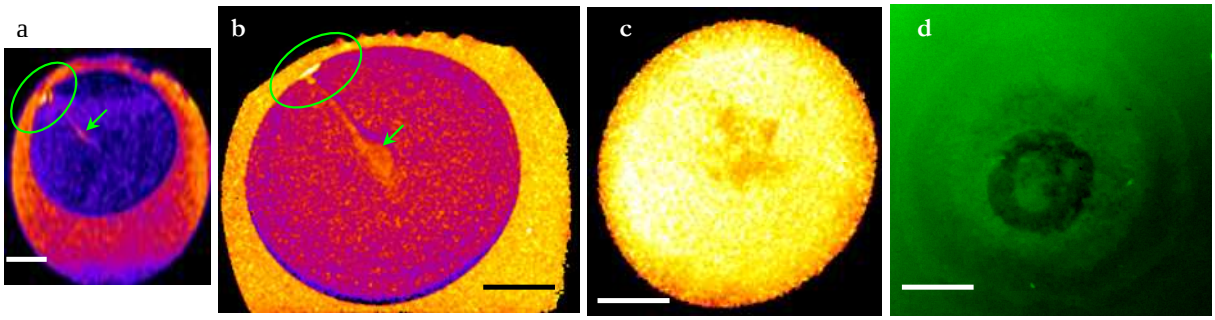

Figure 3.9 : µMRI image of fertilized Quail egg following 24 hrs of incubation.

(a) T_1 image, slice thickness 1mm, pixel size 195 µm × 235 µm; (b) T_2 weighted image, slice thickness = 1 mm, pixel size 98 µm × 98 µm; (c) T_2 weighted image zoomed at the blastoderm, slice thickness 0.5 mm, pixel size 39 µm × 39 µm; (d) optical image of the blastoderm following 24 hrs of incubation. Note, developing embryo is clearly visible as a bright spot at the end of latebra in both T_1 weighted (a) and T_2 weighted image (b). Scale bar: (a-b) = 5 mm, (c-d) = 1.5 mm.

It is clearly visible that the embryo is positioned at the end of the latebra. Image c is the T_2 weighted zoomed image of the 24 hrs old embryo, which can be compared with the optical surface view of the embryo from the top.

3.6.1.3 µMRI image of the quail embryogenesis - after 96 hrs incubation

Figure 3.10 is the µMRI image of a quail embryo after 96 hours incubation. At this stage a very important noticeable aspect in MRI T_2 weighted image is the polarization of the whole egg with very high signal intensity in the upper half and low intensity signal in the lower half which is probably due to the growth of vitalline membrane. The egg yolk

is the lightest part among the egg contents with the blastoderm is the lightest and floats at the top.

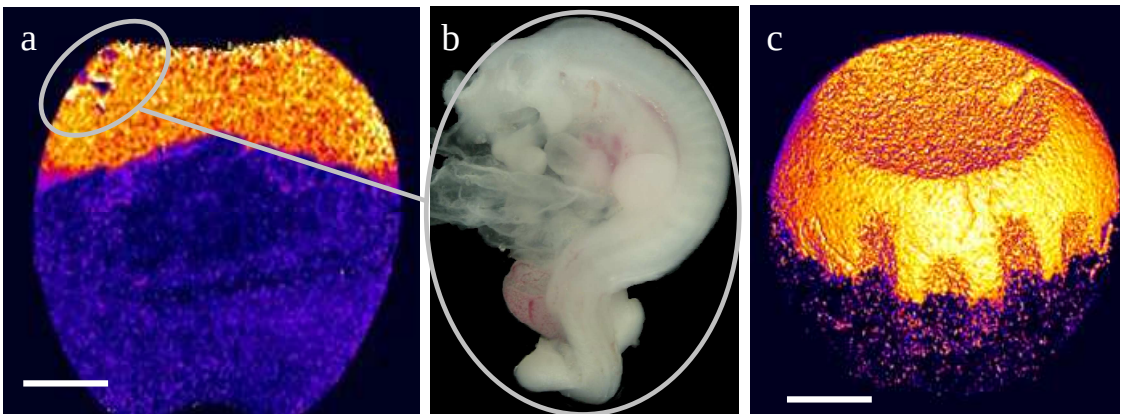

Figure 3.10: µMRI image of fertilized Quail egg following 96 hrs of incubation.

(a) Tomographic T_2 weighted image through the embryo (embryo is marked by the green circle); (b) optical view of a 96 hours old "?" shaped embryo following removal of extra-embryonic membranes, egg white and yolk; (c) 3-dimensional surface reconstruction of the whole egg. Slice thickness 1mm, Pixel size (a) = 195 µm × 235 µm; voxel size (c) = 195 µm × 195 µm × 235 µm. Scale bar: (a, c) = 5 mm; (b) = 1mm.

Inside the egg, the yolk is surrounded by thicker part of egg white and the yolk is suspended in the middle with chalaza. During development, the yolk sac grows and eventually encloses the whole yolk. But the vitelline membrane grows ahead of yolk sac and covers nearly the whole yolk by the end of 4th day of incubation (in case of chicken). Only egg white remains at the lower part of the egg. The whole yolk with growing membrane is seen as very bright object in T_2 weighted image. In the three-dimensional surface reconstruction of MRI data (b in Figure 3.10) this part is seen as solid mass since the signal intensity is very low from the egg white at the lower part of the egg. The embryo is recognized as bright spots in the dark background. At this stage, it is difficult to recognize different parts of the embryo in µMRI image. Eyes of the embryo and abdominal cavity are possibly seen in the image as dark spots.

3.6.1.4 µMRI image of the quail embryogenesis: after 5 days incubation

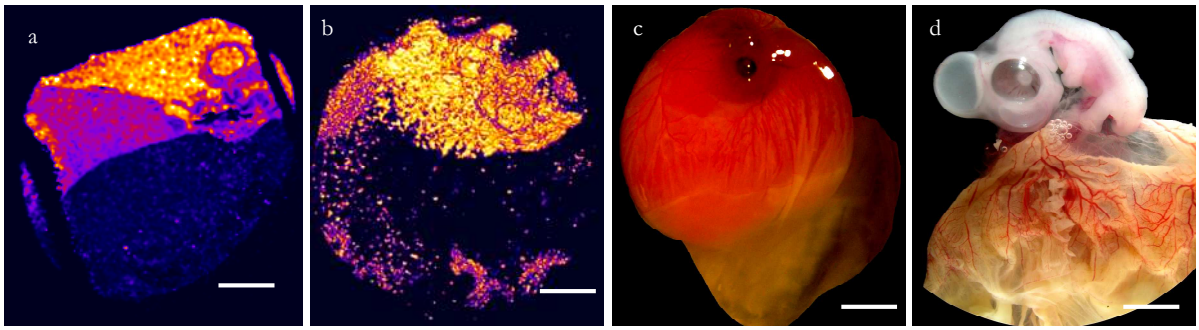

Figure 3.11: µMRI image of fertilized Quail egg following 5 days of incubation.

(a) T_2 weighted MRI tomographic image (coronal section through the head and part of the body). (b) 3-dimentional surface reconstruction of MRI image. Slice thickness 1mm, Pixel size = 195 µm × 235 µm (1); voxel size = 195 µm × 195 µm × 235 µm. (c-d) optical view of a 5 days old quail embryo; (c) along with membranes and yolk following removal of the shell and (d) embryo along with yolk sac following removal of yolk. Scale bar = 5 mm.

Figure 3.11 shows the µMRI image of fertilized Quail egg following 5 days of ncubation. Image (a) is the T_2 weighted image of the embryo, longitudinal section through the body (section through the sagittal plane); image b is the three-dimensional surface reconstruction of µMRI data. At this stage of development, the embryo outline can be well recognised in MRI image. The eyes, head, body contour, abdomen can be clearly seen.

Since the embryo is closely associated with extraembryonic membranes and yolk, and they have MRI signal of similar or closer intensity, it was difficult to distinguish clearly between the embryo and extraembryonic membranes. For this reason, the embryo image could not be reconstructed perfectly from three-dimensional MRI data. In the optical image [image (c) and (d)], it is clearly visible that the embryo is closely associated with the extraembryonic membranes [image (c) is taken after removal of the embryo from eggshell. In image (d), the yolk and the egg white have been removed to expose the embryo optically].

3.6.1.5 µMRI image of the quail embryogenesis: after 6 days incubation

Figure 3.12 shows the µMRI image of quail embryo after 6 day incubation. At this developmental stage, the embryonic parts have become more distinguished. In image a, (T_2 weighted MRI image) mid sagittal section through the body shows different

structures. The eyeball, head, beak, thorax, abdomen, vertebral column are clearly visible. In coronal section through the head shows (image c) shows both the eyeball as bright structure. In sagittal section through the mid body plane (image e) shows the vertebral column and the trunk (thorax and abdomen).

Three-dimensional surface reconstructions of µMRI data (image b) did not provide any better quality image of the embryo at this stage. Image (d) is the optical image of the embryo after removal of the extra-embryonic membranes for better visibility of different anatomical structures. Advantage of MRI in this regard is that it is not required to sacrifice the embryo to see the internal organs. Actually, the whole embryo is imaged *in ovo* in its natural habitat.

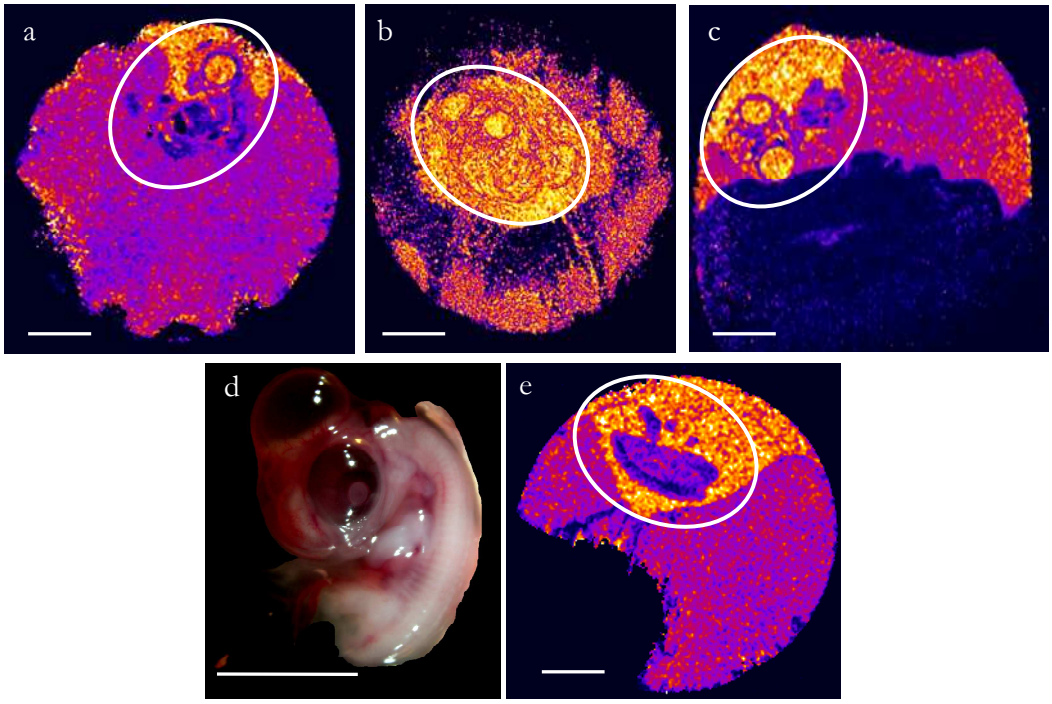

Figure 3.12: µMRI image of the fertilized Quail egg after 6 days incubation.

(a) T_2 weighted MRI tomographic image (longitudinal section through the head and part of the body); (b) 3-dimentional surface reconstruction of MRI image. (C) T_2 weighted MRI image-coronal section through head and part of the body. (d) Optical view of the 6 days old quail embryo. (e) T_2 weighted MRI image: longitudinal section along vertebral column. Slice thickness 1mm, pixel size = 195 µm × 235 µm (a, c, e); voxel size = 195 µm × 195 µm × 235 µm. Scale bar = 5 mm.

3.6.1.6 µMRI image of the quail embryogenesis: after 12 days incubation

Figure 3.13 shows µMRI imaging of a 12 days old quail embryo inside egg. At this stage, the embryo is fully developed and different anatomical structures are well recognised in MRI image. Quail embryos hatch after 17 days of incubation. At this stage

(Incubation Day-ID 12), the embryo is growing and maturing for hatching. In MRI T_2 weighted image, internal body organs can be well recognised. Image (a) in Figure 3.13 which is coronal section through head and body trunk, shows the eyeball, optic lenses, beak, abdominal organs, cross-section of limbs well are recognised. In mid sagittal section (image b and c) shows well-developed skull, vertebral column, thoracic viscera like lung and heat, abdominal viscera can be recognised very well.

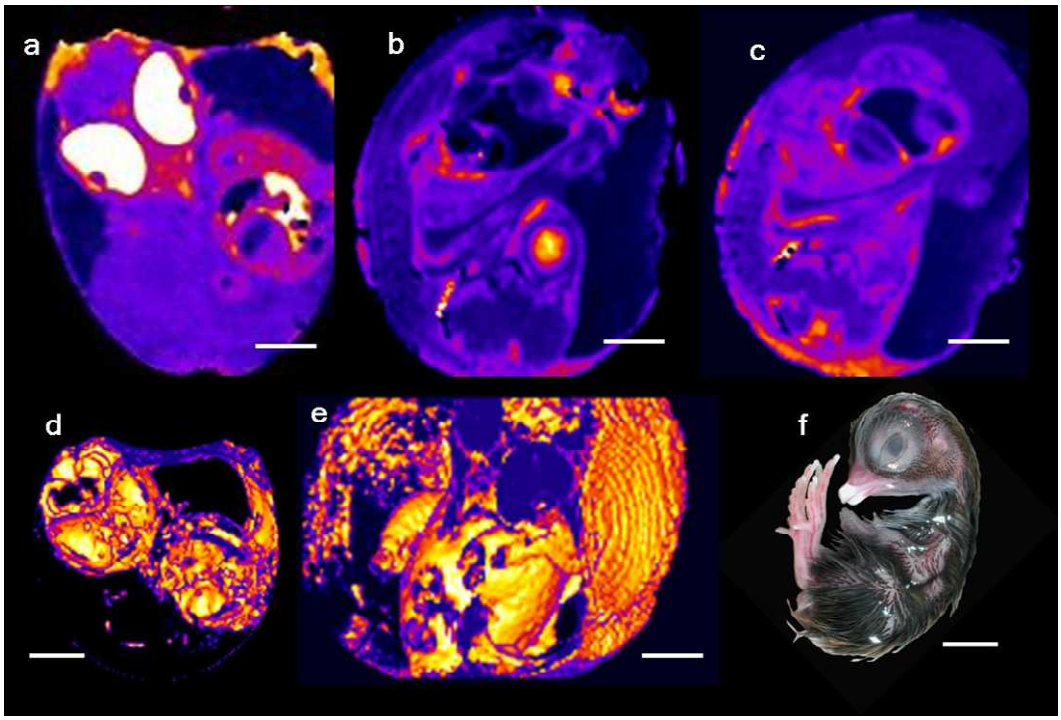

Figure 3.13: µMRI image of the fertilized quail egg at ID 12.

(a) T_2 weighted MRI tomographic image (coronal section through the head and part of the body); (b, c) coronal section through the head and spine); (d, e) 3-dimentional surface reconstruction of MRI T_2 weighted image. Slice thickness 195 µm, Pixel size (a, c, e) = 195 µm × 235 µm; voxel size = 195 µm × 195 µm × 235 µm. Scale bar = 5 mm. (f) optical view of the 12 days old quail embryo.

In three-dimensional surface reconstruction of T_2 weighted MRI data (d, e in Figure 3.13) shows the well-developed embryo with adult contour. Especially in image (e), the body trunk and head are well recognised with limbs. In image (d), the eyeball, part of skull and abdominal organs are well recognised. For comparison, optical view of the same embryo image (f) is shown in relatively same posture which can be very well compared with the three dimensional surface reconstructed data in image (e).

Figure 3.14 shows the comparison between MRI and optical imaging of a quail embryo at ID 12 showing different external body parts. Image (a) is the three-dimensional surface reconstruction of T_2 weighted MRI image inside an intact egg with voxel size = 195 µm × 195 µm × 235 µm. Image (b) is the optical image of the embryo following removal of the extra-embryonic membranes. It is possible to acquire similar quality image with MRI without even disturbing the embryo in its natural habitat. Since the embryo is very closely associated with the extra-embryonic membranes, egg yolk and egg white, it is very difficult to observe the embryo optically. Therefore, the egg must be opened and embryo must be freed and cleaned of the membrane and egg white before it can be optically imaged.

3.6.1.7 Comparison between µMRI and optical imaging: comparison of the embryo externally and internally following dissection

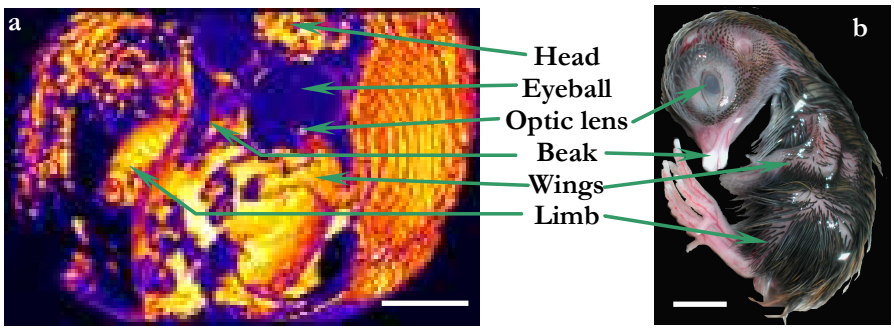

Figure 3.14: Comparison between the MRI and the optical image of 12 day old Quail embryo.

(a) 3 dimensional surface reconstruction of T_2 weighted MRI image (voxel size = 195 µm × 195 µm × 235 µm), (b) optical image. Scale bar = 5 mm.

Figure 3.15 compares between different internal organs and viscera in MRI image and in optical image following dissection. In image (a), green arrow marks the vertebral column (T_2 weighted image) which is shown in the optical image following dissection in image b and c (zoomed) marked by green arrow.

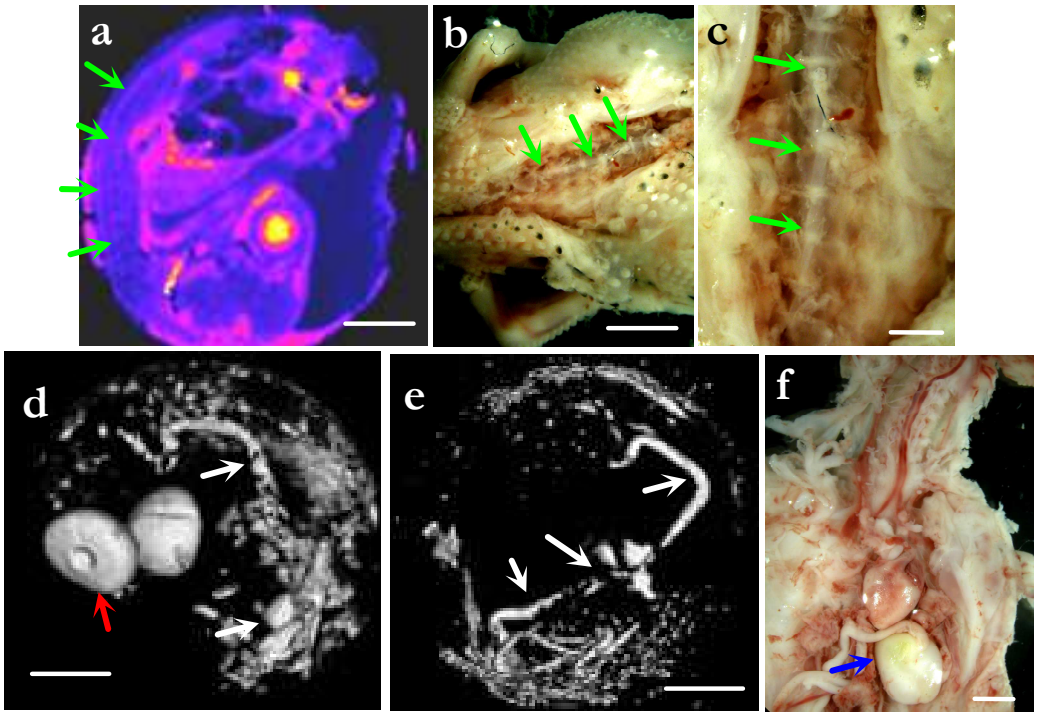

Figure 3.15: Comparison between the µMRI and the optical image following dissection of a quail embryo at ID 12.

(a) T_2 weighted MRI image through the mid sagittal plane (pixel size 195 µm × 195 µm, slice thickness 235 µm). (b) & (c) optical image of vertebral column on dissection (green arrow shows vertebral column). (d) and (e) 3-dimentional surface reconstruction of internal organs: (d) T_2 weighted and (e) proton density image). White arrow indicate Gastro-intestinal tract, red arrow is eyeball and blue arrow in image (f) indicates gizzard. Scale bar (a, d, e) = 5 mm, (b) = 2 mm & (c) = 1 mm.

In three-dimensional surface reconstruction of T_2 weighted MRI data (d in Figure 3.15) shows eyeballs and gastro-intestinal tract that have higher water content. Note the eyeball in image (d); the optic lenses have low water content and shows as an indentation in the eyeball. 3-dimensional reconstruction of Proton density (I_0) image (a-b in Figure 3.16) shows the intestinal tract having higher water content (blue in image a]. Other parts of the embryo show containing less water. In image (b) structures outlined in yellow have higher water content.

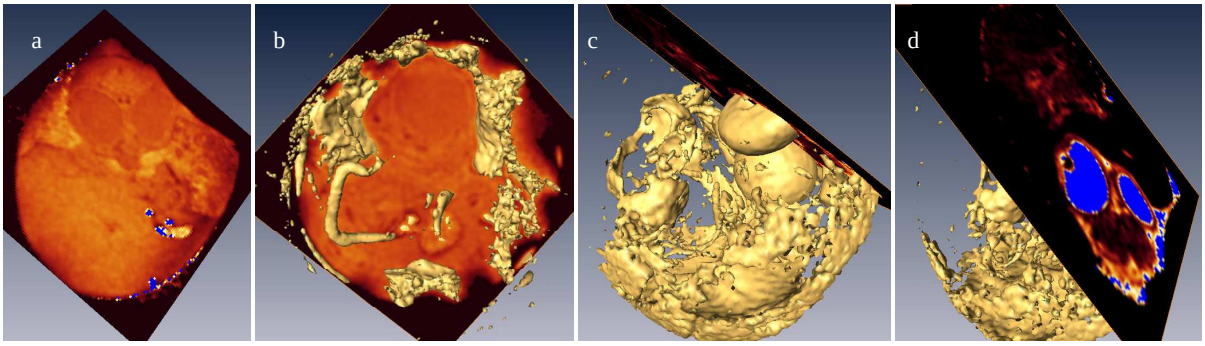

Figure 3.16: Three-dimensional surface reconstruction of the proton density (I_0) image (a-b) and T_2 weighted image of a 12-day-old quail embryo inside the egg.

The image (a-b) shows the distribution of water (proton) inside the eggshell during development. Blue represents higher water content. It is important to note that in the embryo the organs with highest water content are the intestinal tract, which can be better recognised in image (b). Other parts of the embryo have water content in lower extant. In image (a), other parts of the embryo like the head, eyes can also be recognised. Image (c-d) is three-dimentional surface reconstruction of T_2 weighted image.

CAM serves as the organ for gaseous exchange for the growing embryo. It is a highly vascular organ covers the whole inner surface of the egg membrane.

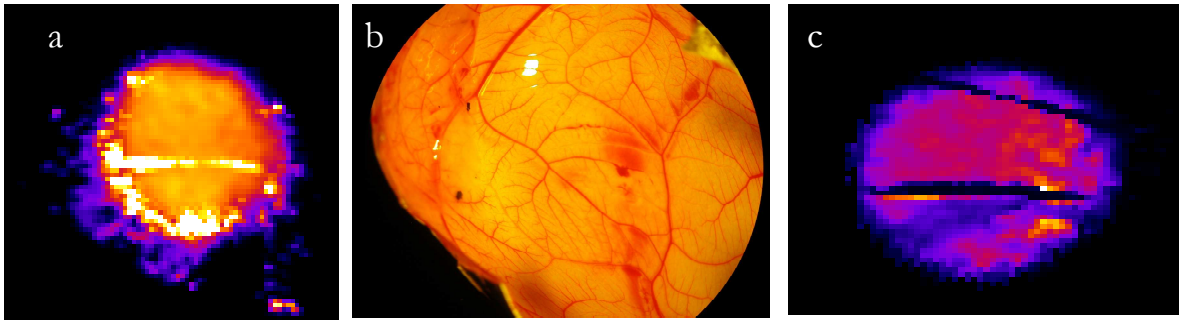

Figure 3.17: µMRI image of extra-embryonic blood vessels.

Chorio-alantoic vessels located at inner side of the shell membrane. (a) Proton density (I_0) image; (b) optical view of extra-embryonic blood vessels located in the inner side of the eggshell; (c) T_2 weighted image. Note in I_0 image, the blood vessels appear bright, in T_2 weighted image appears dark.

3.6.2 Magnetic resonance spectroscopy of egg white

Magnetic resonance spectroscopy of thin and thick egg white with Magic Angle Spinning (MAS) NMR spectroscopy (Figure 3.18) shows the ^{1}H (left) and ^{13}C (right) spectrum of egg white. ^{1}H (left) spectrum, thinner part of egg white has higher water content than the thicker part. Water integral for thin egg white is 8.5; on the contrary, the thick egg white has water integral 7.9. In ^{13}C spectrum (right) of thick and thin egg white shows roughly similar pattern of spectrum in both the egg whites.

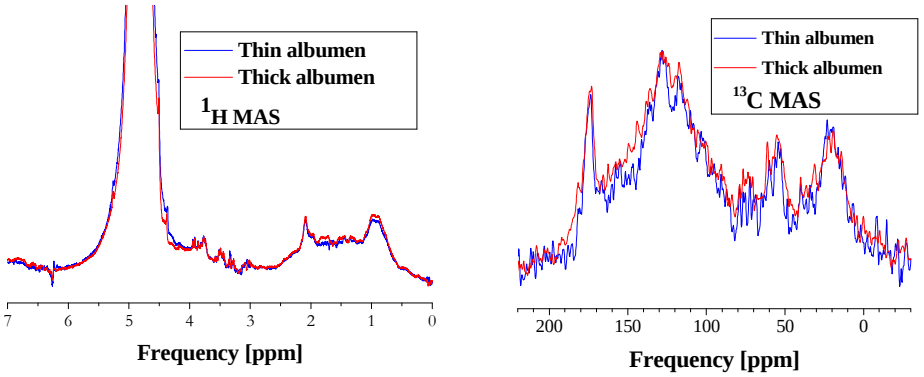

Figure 3.18: Magic Angle Spinning (MAS) NMR spectroscopy of the thin and thick egg white.

The chemical composition is same for both thick and thin egg white, but only water content of the thin egg white higher than thick egg white (water integral for thin egg white: 8.5, thick egg white: 7.9).

The chemical composition of the thick and thin albumen is similar [64] but the thick albumen is richer in ovomucin and ovomucin is responsible for the elevated viscosity [319]. Moreover, the specific gravity of thin albumen is lower than that of the thick albumen and also than that of egg yolk.

3.6.3 Labelling of the stem cell with SPION

Rat pancreatic stem cells (Z29 P19) are cultured with different concentration of FluidMAG-CMX/E (carboxymethyl-cdextran) Magnetic separators MagnetoPURE magnetic plate (ChemiCell GmbH, Berlin, Germany) for different period of time and labelling efficiency was checked with MRI imaging. Cells were cultured with 30 ml PBS (Phosphate Buffer Saline) in T175 flask of confluent cells (5-7e6 cells). The aim was to find out the optimum concentration of SPION and optimum incubation time. Figure 3.19 is T_2 weighted MRI image of rat pancreatic stem cells (Z29 P19) immersed in Culture medium labelled with FluidMAG-CMX/E (carboxymethyl-dextran) in a 4 mm inner diameter glass tube. Cells were centrifuged before imaging. Cell labelling and staining was performed by Dr. Erwin Gorjup from workgroup "Cell Biology and Applied Virology" of the Fraunhofer IBMT.

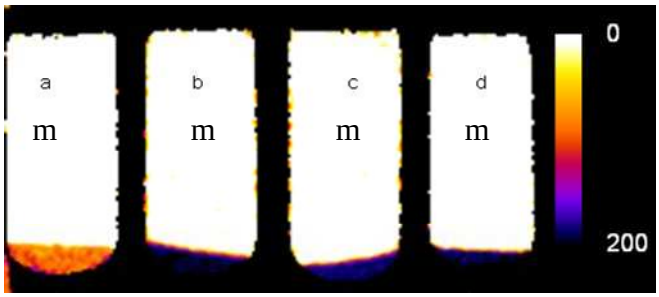

Figure 3.19: T_2 weighted MRI image of the rat pancreatic stem cells (Z29 P19) incubated with different concentration of SPION.

T_2 weighted MRI image of Rat pancreatic stem cells (Z29 P19) immersed in culture medium (m) labelled with FluidMAG-CMX/E (carboxymethyl-dextran) in a 4 mm inner diameter glass tube. Cells were centrifuged before imaging. (a) Control; (b) 16 hrs incubation with 1mg CMX; (c) 1hr incubation over magnetic plate with 1mg CMX (1 hr post magnetic incubation without magnet); (d) 1hr incubation over magnetic plate with 5mg CMX From chemicell GmbH, Berlin, Germany)

Figure 3.20 shows rat pancreatic stem cells (Z29 P24) labelled with SPION without lipofection, injected into an agarose phantom (0.025% by weight) and imaged with µMRI to check the cell labeling efficiency. It is important to note that T_2 weighted value of labelled cells incubated with SPION with or without magnetic plate were ≈120 ms whereas T_2 weighted value of egg yolk was much lower, ≈30 ms. This shows that the labelling was not efficient enough for tracking labelled cells inside the egg.

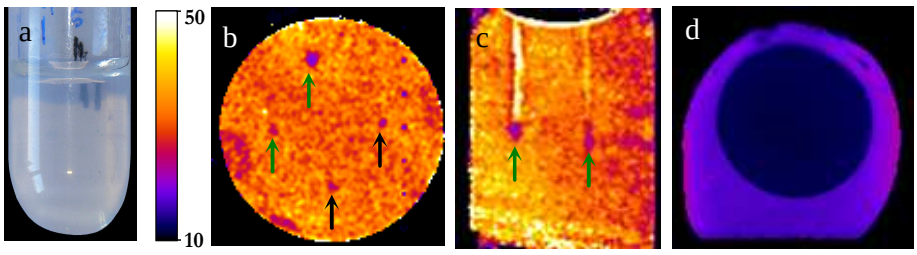

Figure 3.20: µMRI image of the SPION loaded rat pancreatic stem cells (Z29 P24) without lipofection.

Rat pancreatic stem cells (Z29 P24) were incubated for 3.5 hrs on magnetic plate with FluidMAG-CMX/E (carboxymethyl-dextran) 1mg CMX that was injected into an agarose phantom (0.25% by weight). Amount of cells per injection: ≈10,000-20,000 cells. (a) Optical view of the agarose phantom; (b, c) MRI T_2 weighted image. (b) is cross sectional view, (c) is longitudinal section of the agarose phantom at the label of injected cells. (d) T_2 weighted MRI image, longitudinal section of a quail egg injected with SPION labelled cells with incubation over magnetic plate. T_2 weighted value of labelled cells are ≈120 ms (T_2 weighted value of egg Yolk is ≈30 ms). Arrows indicate labelled cells (green arrow indicate ≈20,000 labelled cells, black ≈10,000)

Rat pancreatic stem cells (Z29) were labelled with SPION with lipofection and different amounts were injected into a fertilized quail egg through a small hole at the blunt end of the egg. The labelled cells were injected inside egg white avoiding egg yolk, because small puncture in the egg yolk may cause yolk leakage. For control,

nonlabelled cells were also injected inside the egg. The cells were centrifuged before taking the sample so that the cell bolus contained as little culture medium as possible. The location of the injection was marked with small capillary tubes filler with copper sulphate solution attached near the location with adhesive tapes.

3.6.4 Cell labelling with the SPION using lipofection technique

Lipofection

Figure 3.21 shows rat pancreatic stem cells (Z29) incubated with SPION (CMX from chemicell GmbH, Berlin, Germany) and stained with Prussian blue for visualization of intracellular Iron (Fe). Iron is stained blue in this staining method. Visual impression of the image shows that lipofected cells [image (c, d)] have higher Iron load than non-lipofected cells [image (a, b)]. Quantitative estimation of cellular Iron content was not performed.

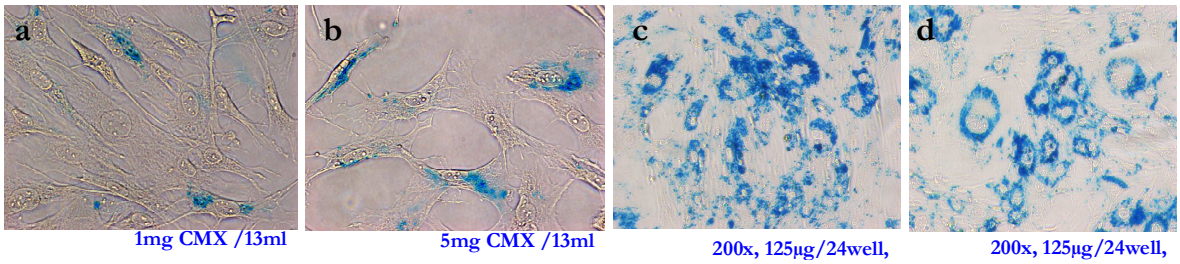

Figure 3.21: Cell labeling efficiency of SPION with lipofection: SPION labelled cells stained with Prussian blue.

Rat pancreatic stem cells (Z29) incubated with CMX and stained with Prussian blue for visualization of iron (Fe). (a-b) Without Lipofection; (c-d): following Lipofection (c) Lipofectamin, (d) Optifectamin. Visual impression of Prussian bluestained cells show higher iron load in lipofected cells than non-lipofected ones.

3.6.5 Imaging of the SPION into the fertilized quail eggs

25 µl SPION was directly injected inside a fertilized quail egg and tracked with MRI imaging over 24 hours. The aim was to observe the contrast, fate and intensity of SPION itself. Figure 3.22 shows the T_2 weighted MRI image of a fertilized quail egg injected with SPION over 24 hours.

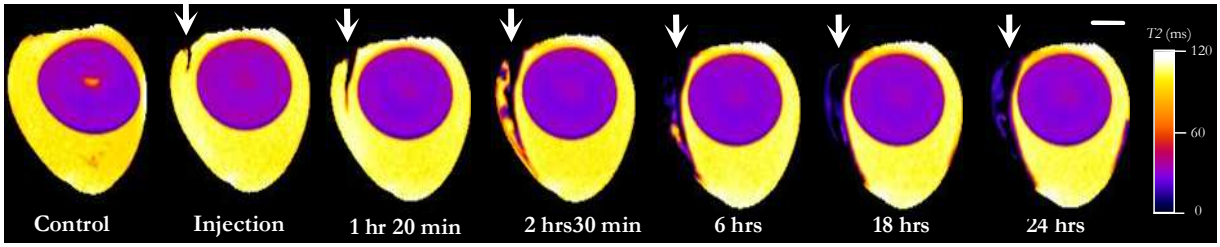

Figure 3.22: µMRI T_2 weighted image of the SPION injected into fertilized quail egg.

Amount injected was 25 µl. (Scale bar = 5mm).

The SPION was injected through a small hole at the blunt end of the egg. The SPION sank vertically to the bottom of the egg through the egg white. After 18 hours of injection, the SPION reached the bottom of the egg. Gradually it diffused little to the side and at the end of 24 hours; the whole track of SPION movement could be detected.

3.6.6 Imaging of the SPION labelled stem cells with lipofection injected into the fertilized quail eggs

Figure 3.23 shows the T_2 weighted MRI image of the fertilized quail egg after injecting different amount of SPION labelled rat pancreatic stem cells (Z29).

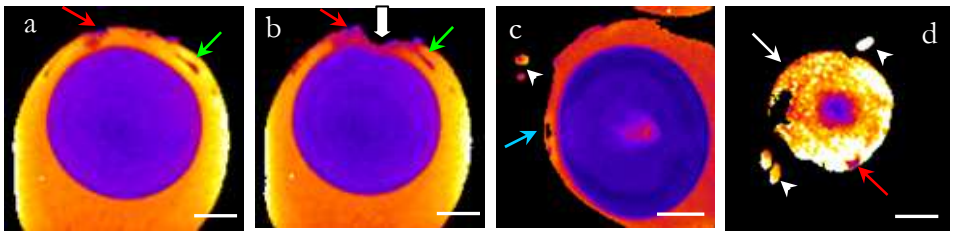

Figure 3.23: µMRI T_2 weighted image of CMX labelled stem cells injected into fertilized Quail egg.

MRI T_2 weighted image of CMX (Chemicell GmbH, Berlin, Germany) labelled stem cells injected into fertilized Quail egg. (a-b): T_2 weighted MRI image of a quail egg injected with nano iron particle along longitudinal axis through the injected cell site. (a, c, d): immediately following injection, (b) 20 hrs later. Amount injected: green arrow: 100 µl, red arrow: 75 µl, blue arrow = 25 µl labelled rat pancreatic stem cells (Z29 P24), white arrow = 25 µl non-labelled cells as control indicated at the site of injection. Note that it is possible to detect as small as 25 µl cell bolus. White arrowhead marks the capillary tubes to localise the site of injection. Scale bar = 5mm

In this experiment 100, 75 and 50 µl cell bolus were injected into the quail egg. It was possible to detect as little as 25 µl cell bolus with MR imaging. The fate of the injected cells was also followed over 20 hours. The cells remained nearly at the same position. During this experimental procedure, eggs were not incubated.

3.7 Discussion

In ovo µMRI can monitor the process of embryonic development noninvasively with very high resolution. Having the advantage of non-invasiveness, µMRI ease the process of the whole study where the tedious and time consuming work of sample preparation is not required. The avian embryo has long been an experimental model to study the process of embryonic development. Traditionally this is done by opening the incubated egg at different stage of development and observing the embryonic stages of development.

For µMRI experiments with avian embryo, quail eggs were selected. Because the maximum size of the sample possible to image inside the Bird cage imaging coil was 25 mm. Quails lay small eggs (≈15 g) having short axis diameter about 20-25 mm which fits very well into the birdcage imaging coil. Also quail has relatively short incubation time (17 days) which shortens the experimental procedure.

Traditional methods observe the dead embryo at different embryonic developmental stages. However, the actual *in ovo* vital process remains obscure. µMRI offers live *in ovo* real-time imaging at very highe resolution. Being enclosed in hard, calcareous, opaque shell, avian eggs are inappropriate for other types of imaging. In µMRI the magnetic field is not obstructed and shielded by the calcareous shell. The vital process of embryonic development can be observed from outside while the embryo remains undisturbed during the process of imaging or spectroscopy. Since the magnetic field is non-ionizing, it has no negative influence on the process of embryogenesis. Teratogenic and mutagenic impact of strong magnetic fields on embryonic development is very low [212, 298, 356].

During the process of oogenesis, the white yolk is produced at night and contains more protein. Most of the white yolk is located directly below the nucleus (the position of the possible future embryo) in the latebra and the nucleus of pander. However, as egg-laying time grows closer, the egg yolk is produced continually and begins to accumulate rapidly. For the last seven or eight days before the ovum is ovulated, yellow and white yolk are laid down in rings like those of a tree. When the ovum has accumulated enough yolk to grow a chick, it is released from the ovary. In the zoomed µMRI view of the blastoderm in image c shows the blastoderm cells as bright ring. Yolk

fat is suppressed in inversion recovery imaging sequence. The image (d in Figure 3.7) is the optical view of the blastoderm surface placed beside the MRI image for comparison.

Transplantation of stem or progenitor cells is a revolutionary new technique proposed for the treatment of various injured tissues or organs. Because cells can be isolated from different tissues, expanded *in vitro*, and replace or repair defective endogenous cell populations [170, 206, 327, 343], they offer new promise for tissue repair and disease correction. Cell therapy appears a promising field for the treatment of human diseases. As part of this new field, transplantation of smooth muscle cells has undergone extensive investigation in recent years as a potential therapy, mainly for repair of aneurysm [8] or myocardial ischemia [399] or for cardiac graft [245]. Lack of ability to track the cell transplants, however, remains a major problem that must still be overcome to understand and optimize cell therapy. In fact, most cell transplantation techniques involve the use of histological analysis to evaluate cell transfection, proliferation, and migration [166, 243, 341]. Yet, following the status of stem cells *in vivo* is critical if therapy is to be optimized or evaluated. Because magnetic resonance imaging (MRI) allows for three-dimensional (3D), high-resolution, whole body imaging and tracking of cells *in vivo*, it is an ideal technique for determining the fate of cells after transplantation and migration of cells after injection, without the need for tissue biopsies and histological assessment. However, following cells by MRI requires that they contain paramagnetic probes.

Molecular imaging by chemically modified of SPION surfaces by attachment of functional groups and further covalent coupling with biodegradable substances can help in precise target oriented study of cellular process. Differentiation of normal vs. neoplastic liver tissue is currently the most significant application in the use of SPION, which resulted in the 1996 FDA approval of the product Feridex®. The long-term viability, growth rate, and apoptotic indexes of the labelled cells were unaffected by the endosomal incorporation of SPION, as compared with these characteristics of the nonlabelled cells. In nondividing human mesenchymal stem cells, endosomal iron nanoparticles could be detected after 7 weeks; however, in rapidly dividing cells, intracellular iron had disappeared by five to eight divisions [17].

The SPION are one of the novel methods used in clinical hepatic MRI imaging, tumour imaging and many more. During the last few years, clinical trials have begun to evaluate the use of stem cell transplantation for tissue and organ repair. Yet, no good

non-invasive method exists for following cells *in vivo*. Clinically approved cell labelling with iron could mean a breakthrough in stem cell transplantation, because it allows potential monitoring of the biodistribution of cells over time with MRI and therefore may provides a means to track cellular fate. In monitoring transplanted cells *in vivo*, the MR signal intensity and corresponding T^1, T^2, and T^2* values depend on two distinct, relevant parameters: first, the density of labelled cells in a given volume, and, second, the intracellular particle load in labelled cells. Both parameters are involved in the proliferation and migration of transplanted cells within the targeted organ.

Real-time *in ovo* imaging of cellular process is a great challenge. In the early stage of development, the embryo is optically transparent; which may allow optical imaging in conjunction with fluorescently labelled cell to follow in the embryo in an open system of avian culture (Chapter 5). Nevertheless, as the embryo grows, it becomes opaque and application of optical methods to monitor cellular processes inside the body deep into an organ is not possible. MRI can play a key role in this field with SPION as contrast media. Taking the advantage of unique "proton" stains, non-destructive, inherently 3-dimensional nature of MRI can image the internal organs with a very resolution. Applying SPION labelled cells as contrast media can reveal vital processes of life at or near cellular level.

One drawback of MR imaging is motion artefacts. The movement of the imaged object or a part causes motion artefacts during the imaging sequence. The motion of the entire object during the imaging sequence generally results in a blurring of the entire image with ghost images in the phase encoding direction. Movement of a small portion of the imaged object results in a blurring of that small portion of the object across the image. As the embryo grows, it starts to move in the amniotic cavity. The heart starts to beat at the end of first day of incubation. Gradually from day 4 of incubation, the movement of the embryo increases. In later stage, the embryo actually swims in the amniotic fluid. In clinical MR imaging of the thoracic cavity including heart, quicker imaging sequences are employed triggered by ECG so that the image can be acquired at the same state of cardiac cycle. However, the embryonic movements are irregular and solving this problem was difficult. It is even worse in high-resolution imaging. Since the image acquisition time of the MR imaging relatively long compared to other imaging methods, it was very difficult to obtain high-resolution images.

Motion artefacts are very important obstacles of MRI regarding imaging the developing embryo. To overcome the problem of motion artefacts, the embryo was cooled down to 4 °C with the flow of N_2. Even with many advantages over other imaging methods, µMRI was not a suitable tool for *in ovo* real time imaging.

The signal-to-noise ratio (SNR) of an MR experiment shows – both in the time and the frequency domains – a very complex dependence on the characteristics of the static field, the rf circuitry, the signal acquisition and transformation techniques applies to frequency domain SNR) and, of course, the sample [130]. For the purposes of this thesis, the most important relationships can be summarised as follows:

$$SNR \propto \frac{V_S . \omega_O^{7/4}}{T_S . T_C^{1/2}} \qquad \text{Equation 3.7}$$

where Vs is the sample volume, while Ts and Tc represent the temperatures of the sample and the rf coil, respectively, whose inverse relationships with SNR are good news for cryoapplications, as they can partly compensate for the impact of low temperatures on the population difference (cf. Equation 3.2) but bad news for higher temperature application. Besides the sample volume Vs is directly proportional to the SNR. That means, in high-resolution imaging, the sample size is smaller and the SNR worsens further. To compensate that, signal has to be accumulated for longer period. This means more accumulation motion artefacts during longer acquisition. To freeze the embryonic motion, the egg containing the embryo was cooled down to 4 °C with the flow of N_2 for an hour to kill the embryo before image acquisition.

The MRI micro-imaging system used in this experiment Bruker DMX 400 spectrometer with micro 2.5 gradient system and 9.4 T superconducting vertical bore magnet (He/N_2 cooled) from Bruker BioSpin equipped with 1T/m gradient system had smaller imaging coils and allowed maximum size of the probe of 30 mm X 30 mm X 30 mm. In this small space, it was not possible to construct an incubator maintaining 37.5 °C temperature, 65% RH and rocking of the sample for continuous *in ovo* observation. The existing thermoregulation provided with the system uses liquid N_2 for cooling. Gaseous N_2 evaporated from fluid N_2 is heated with a heating coil and maintains a desired temperature through a close loop thermoregulation system. The N_2 inlet was exchanged with compressed air, but it was not possible to reach 37.5 °C. Besides the

thermal sensor is located directly above the heating coil and shows higher temperature. The imaging coil is located at a bit higher position and the thermal sensor is showing nearly 5°-10° higher temperature than the actual temperature of the probe. Since there is very high magnetic field inside of the magnet (9.4 Tesla), precaution had to be taken during use of other instruments. Beside, instruments may not function perfectly in high magnetic field; especial types of instruments suitable for this purpose had to be used. Using extra thermometer probe to measure the actual temperature of the sample showed thermal difference of 5-10 °C between the probe and measuring system and it was not possible to further raise the measuring probe temperature. Using higher target temperature in the system also could not solve the problem. In addition to reach higher target probe temperature, it is necessary to pumps more air to make thermal equilibrium, which increases the possibility of shaking and vibrating the sample and eventually blow the sample out of the imaging coil. To attain humidity, the air stream was pumped through water bath heated at 37.5° C. Desired humidity was attained. Since relative humidity is dependent on the temperature, humidity attained by the mentioned method was much higher approaching 90-100%. Since the temperature was low, there was much condensation in the measuring sample, which further worsened the situation.

µMRI can be combined with other methods for a better view into the cellular process of embryonic development. These will be illustrated in the next chapters where gradual technical modification of avian egg towards a complete artificial egg to use as a system for culture of eukaryotic cells will be carried out along with development of different techniques to gain information from growing avian embryo will be carried out.

At the end, it can be concluded that µMRI is not a solution to fulfil the final goal of this thesis. It is not feasible for imaging the developing embryo at the cellular level. It is important to understand the cellular microenvironment behind linage specific differentiation *in ovo* which is not possible *in vitro* until now and to fulfil the aim of this thesis to construct an artificial egg for culturing cells *in vitro* at the liquid|liquid interface with known boundary conditions.

3.8 Outlook

The avian embryo has a long history as a model system in developmental biology and has contributed major concepts to immunology, genetics, virology, cancer and cell biology. Traditional study methods were to sacrifice the embryo and make histological section and staining to observe the developmental status of the embryo. However, it is not possible to observe the dynamic processes involved at molecular and cellular level. The traditional cell and tissue culture methods on the contrary, are based on artificial systems developed for microbiological studies. Gradually these two disciplines are getting further apart. Cells divide in culture and produce cells but It is not yet possible to culture tissue-like structures with architecture and different types of cells performing a specific type of task in coordination. But this complex phenomenon is happening inside fertilized eggs where a single cell differentiates into different types and eventually makes a whole animal.

Perhaps µMRI could be complementary to other methods of microimaging methods but alone is not a solution to study rapidly growing and moving embryo. To fulfil the definitive aim of this Ph.D. work-"**construction of an artificial egg**" it is necessary to modify the natural avian egg systematically and to check the functionality. Hatching of a viable bird is set as the parameter to asses the functionality of the modified system. Hatching is an important parameter in this aspect for assesing the functionality of the modified system. Since the avian egg is the result of evolution and gradual perfection for millions of years, a perfect environment for cellular growth, development and differentiation; only that guarantees viable hatchlings. Therefore, to imitate the natural process *in vitro*, it is necessary to asses the functionality of the modified system, and hatching is the parameter. If it is possible to bring viable hatchlings from the modified system, the functionality of the system can be accepted which can be further modified based upon the guideline of hatching.

4 Cultivation of avian embryos in open systems

4.1 Summary

Objective: As described earlier, the avian eggs are independent of mother regarding the logistics for the developing embryo except for the warmth. This make the avian eggs very especial for embryological experiments. Such experiments involving mammalian embryo is impossible since the embryo develops deep inside the body of the mother animal and totally dependent on her. However the opaque, calcareous eggshell of avian egg is an obstacle for conventional imaging. It is therefore necessary to culture the avian embryo in open systems to bring them to hatching. Only the hatching of a viable bird guarantees theoptimum functionality of the open system which can be further modified. It will be then possible to study different cellular processes in detail *in ovo* which is not observed *in vitro*.

The main purpose of the experiments in this chapter was to culture avian embryos in open systems and brings them to hatch. The avian embryo was explanted from the egg and cultured in different open systems and was brought to hatch. The hatching of a viable chick was set as the standard to asses the the system since only hatching guarantees the functionality of the system. The open sysytem can then be modified stepwise towards more artificial and technical system in future where different boundary conditions will be known.

Methods: The explanted avian embryos from different avian species were cultured in open systems, which consisted of surrogate shells taken from larger eggs than the donor and the complete artificial system consisted of Teflon membrane suspended by a stainless steel net inside a glass tube. The open ends of the both systems were covered with double layer of cling film.

Results: Embryos from different avian species were successfully cultured and brought to hatching in surrogate shell open system. In the completely artificial system, no embryo developed until hatching. Because of technical limitations, complete artificial system was only applied to the quail embryos. For the first time, effect and influence of surrogate shell on avian embryo was identified.

Conclusions: The surrogate shell open culture system was found to be effective for further experimentation, since hatching rate were higher in this system provided cultured in appropriate conditions. Bantam chickens were selected as the model animal for further experiments. The hatching rate of the quail in open system was very low, because of the difficulties finding appropriate surrogate shell. This open system is a revolutionary method which allows *in ovo* observation of amazing intravital process live, in real time in a growing avian embryo.

4.2 Materials required for the cultivation of avian embryos in the open systems

Equipments	Manufacturer
Centrifuge, 3K18	Sigma-Aldrich, Deisenhofen
Eierschalensollbruchstellenverursacher	WMF AG, Germany
GG 12 Proxon Engraving tool	Proxon, Germany
Laminar flow cabinet	Heraeus, Hanau
Lumix FZ50 digital camera	Panasonic, Japan
Microscope	Olympus, Hamburg
Pipett	Eppendorf, Hamburg
Refrigerator (4 °C)	Liebherr, Biberach a. d. Riss
Top-Profi 240 egg incubator	Hemel Brutgeräte, Germany
Wather bath	Julabo Labortechnik, Seelbach
176/M2 incubator	Bruja, Germany
Chemicals	**Manufacturer**
Amphotericin B (250 µg/ml)	Fisher Scientific, USA
Bacillol AF disinfections solution	Bode Chemie, Hamburg
Calcium L-lactate hydrate	Sigma-Aldrich GmbH, Germany
Hypochloride solution (200-500 ppm)	VWR International GmbH
P/S Penicillin/ Streptomycin	Invitrogen Corporation, USA
Biological agents	**Manufacturer/supplier**
Fertilised quail eggs	Wachtelei-spezialist, Aletshausen
Fertilised bantam chicken eggs	Anita Nefzger, Leutershausen
Fertilised White Leghorn chicken eggs	LSL Lohmann Tierzucht GmbH. Cuxhaven
Unfertilised broiler eggs	Glückliche Eier, Saarbrücken
Accessories	**Manufacturer/supplier**
Cling film	Lakeland, UK
Disposable syringe	B. Braun, Germany
Diamond cutter disc	Schnarrenberger, Vöhringen
Diamond cutter rotating disc	Proxon, Germany
Eierschalensollbruchstellenverursacher	WMF, Germany
Glassware	Schott, Mainz
MilliWrap	Millipore Corporation, USA
Parafilm	American National Can. Co., Chicago (U.S.A.)
Poultry feed	Wachtelei-spezialist, Aletshausen
Stainless Steel net	Drahtweberei Gräfenthal, Gräfenthal
Surgical instruments	VWR International GmbH
Computer software	**Developer**
SolidWorks	SolidWorks Corp., Massachusetts, USA

4.3 Motivation

As discussed in Chapter 3, µMRI allows very high-resolution imaging of the embryo in closed egg in its natural environment. However, it was limited by longer image acquisition time, motion artefacts, insufficient free space inside the imaging chamber of the µMRI machine and smaller probe size, which made it very difficult to construct an incubator *in situ* for *in ovo* imaging keeping the embryo alive inside the superconducting magnet of the MRI machine. The MRI micro imaging system consisted of a vertical bore superconducting magnet where the imaging probe lies deep inside the magnet from the opening. In addition, very high magnetic field (9.4 Tesla) in the imaging chamber made it difficult for installation of traditional micromanipulation system; which are mostly constructed of magnetic materials. On the contrary, optical imaging methods are very effective offering very high-resolution imaging at the cellular level with ease. In addition, these methods are relatively inexpensive than µMRI. However, the practical problem remains with the hard, non-transparent shells of the avian eggs, which make optical and other imaging methods inapplicable. It was necessary to remove the eggshell tfor imaging. Removing a part of the non-transparent eggshell and observe the embryo through the window would be one of the possibilities. However, the eggshell is tightly packed with its contents and it is quiet inconvenient to work in such system. Moreover, the imaging probe has to be adequately illuminated for optimum imaging which is difficult in windowed egg.

The method of avian culture in open system is not new; it has been used by the biologists to produce transgenic chickens as bioreactors for pharmaceutical protein production in eggs [124, 141]. In the early days, the experiments started with the windowing of the eggshell, which is simple. Transduction of embryos with retroviral vectors [146, 205, 257, 292] through a small window in the eggshell, which was sealed [11, 35, 123, 124, 290, 308] but often results in low hatch rates [308]. Later an improved sealing technique was developed to avoid trapped artificial air bubbles during sealing, resulted in 45% hatchability [11]. Egg-shell windows are tedious to make, often induce inflammation in the CAM [91], and are too small to adequately visualize the activity inside. In contrast to simple windowed eggs, surrogate eggshells provide a potentially better accessibility for the manipulation of the embryos and allow for multiple operations to be performed during incubation [218]. Besides, culture in complete artificial system has also been reported [147]. The basic

method has been modified a little and used for the production of transgenic birds. But there were not much progress regarding the optimisation and use this method for real time observation of the process of embryogenesis and apply the knowledge for *in vitro / in ovo* cell culture. Unlike mammalians, avian eggs are independent of the mother and this makes the egg very especial for such experiments. Such embryological experiments involving mammals would require immense technological support and of course financial involvement to monitor the embryonic process at cellular level real time *in vivo*, since the mammalian embryo develops inside the uterus of the mother animal and totally dependent on her for nutrition, gaseous exchange and all other sort of support.

For experimental purpose, it was necessary to construct a stable and durable system whose parameters and different boundary conditions are known. In case of traditional monolayer *in vitro* culture of cells, boundary conditions are relatively limited. In comparison to the embryogenesis, the embryonic development is very complex and most of the parameters are unknown. Today's avian egg is the gradual result of perfection through the evolution of millions of years. It is quiet easy and logical to study an existing perfect functioning system than to search for something new. The embryonic stem cell differentiation in *in vitro* culture is uncontrollable and the process is more or less stochastic. Nevertheless, the same cells *in ovo* differentiate into all different types of cells and eventually form a complete viable organism after a period of 21 days of incubation (for chicken). It is very important to find out the boundary conditions *in ovo* for targeted *in vitro* stem cell differentiation into desired cell type and for tissue engineering. Since the viable hatchling is set as the evaluation standard to check the durability of the constructed system, it was very important to characterise different parameters of the constructed system, which can be used as parameters for further experimentation.

4.4 Review of previous works

The culture of the explanted chicken embryo has long been an elegant method to study the early development [237]. The embryonic lifespan of the chick is 22 days. Development in the first day takes place in the oviduct and in the remaining 21 days in the shelled egg. The development of chick embryo had been divided into three periods: fertilization to blastoderm formation (stage I) which lasts for one day [165], embryogenesis

(phase Ⅱ) lasts for three days and embryonic growth (phase Ⅲ) for rest eighteen days [113]. Fertilization takes place in the anterior oviduct, after which the yolk-laden ovum is encased in albumen secreted by the magnum. Around the time of the first division of zygote takes place, some 4.5 hours after ovulation, the shell membrane is deposited in the isthmus and the albumen is doubled in volume by the absorption of uterine fluid. In the final 18 hours of the oviductal phase, the shell is calcified. The second and third phase takes place in shelled egg. There had been attempts to culture both oviductal [237] and postlaid embryos.

Use of polyethylene bags has permitted development of the postlaid embryos for 10 to 12 days [81, 296] and modification involving especially constructed glass dishes in combination with polyethylene bags have permitted some survival up to 16 days [58]. Especial incubators were constructed to improve the embryo explants [269]. Shell less culture allows continuous observation and access to the embryo. In 1974, Auerbach et al [20] developed methods for culturing chicken embryos in Petridish with 1-2% CO_2 mixed with air. Dunn, Dugan and Ausprunk also experimented in this field [21, 72, 74]. Rowlett and Simkiss [288] first reported that 3-day incubated chicken embryos inside the egg could be cultured to hatching using turkey eggshells.

Then, Perry [255] devised a complete three-step culture method for the chicken embryos from the single-cell stage obtained from the posterior region of the magnum to hatching using glass jars and chicken eggshells. In this method (system Ⅰ), the germinal disc was covered with a dense albumen capsule. This embryo culture system was applied for culturing early cleavage stage embryos (stage Ⅱ) [87] obtained from the anterior region of the uterus by hormonal treatment [224]. Naito et al. [222] found that a dense albumen capsule surrounding the ovum was not essential for the normal development of the embryos from stage X (System Ⅰ) [87] onward and could be substituted by thin albumen. Based on this discovery, they devised a modified method for culturing chicken embryos from the single-cell stage to hatching, that is, removing the dense albumen capsule from the stage X onward (systems Ⅱ and Ⅲ), and achieved a high rate of hatching (34.4%). After that, Naito et al. [223] succeeded in culturing the chicken embryos obtained from the anterior region of the magnum to hatching. In this case, a thin layer of dense albumen capsule was formed around the ovum, and the germinal disc region of the ovum was covered with gauze. Kamihira et al. [147] succeeded to culture

quail embryo in chicken surrogate shell and complete artificial system with viable hatchlings. They preincubated the embryos inside the egg for two days. Than quail embryos were transferred to a complete artificial system consisted of porous Teflon membrane suspended on a stainless steel mash inside a glass tube with holes. They used calcium supplementation for shell less culture (calcium lactate or egg shell powder).

4.5 Experimental approaches

As described earlier, the embryonic lifespan of the chick is 22 days. Development in the first day takes place in the oviduct and in the remaining 21 days in the shelled egg. By the time the egg is laid, the embryo has already reached the blastoderm stage. Here in this chapter and rest of the thesis, the experiments were designed for the cultivation of the chicken and quail embryos only from the blastoderm stage to hatching (postlaid eggs) in surrogate shell and complete artificial system. Due to technical limitations, the complete artificial system was only applied to the quail embryo. Embryos were handled according to the guidelines of German animal protection laws [Tierschutzgesetz (TierSchG) in der Fassung der Bekanntmachung vom 18. Mai 2006 (BGBl.IS. 1206 ff. ber. S. 1313) und dem Gesetz über das öffentlische Veterinärwesen und die amtliche Lebensmittelüberwachung (VetALG) vom 19.05.1999 (§ 1 Abs. 3 Amtsbl. S 844, 851)].

4.5.1 Culture of the avian embryos in surrogate shells

For cultivation of avian embryo in surrogate shell, the embryo was harvested from the egg after preincubation (72 hrs for chicken and 48 hours for quail) and explanted into a a surrogate shell prepared from larger eggs for further incubation. The opening of the surrogate shell was closed with a double layer of cling film (from Lakeland, UK) and. further until hatching.

4.5.1.1 Preparation of the donor embryos

Donor embryos were prepared from commercially available fertilized chicken (*Gallus gallus domesticus*) and quail (*Coturnix coturnix*) eggs. For good fertilization, least male to female ratio of 1:6 were ensured. For chicken embryos, bantam and White Leghorn strain and for quail embryos, commercially available breed in Germany were

chosen. On arrival, eggs were cleaned and disinfected with hypochlorite solution (Annexes) and stored in a refrigerator for 24 hours at 14 °C. Eggs with cracked and irregular shells, rounded contour and too large or too small size were discarded. On the following day, eggs were placed inside a forced air incubator at 60% relative humidity, 37.5 °C temperature and 90° side to side rocking for 72 hours (48 hours for quail).

4.5.1.2 Preparation of the surrogate shells

Selection of the surrogate shells

As described in section 4.4, explantation culture of avian embryo in surrogate shell is an established method for production of transgenic birds. There had been some experiments involving culture of different avian species like quail embryos in chicken egg shell [147, 150, 239, 240, 242] or chicken in turkey (*Meleagris gallopavo*) egg shell [34, 288] but these data could not be used generally for other species of birds. Especially to find matching surrogate shell for the donor embryo for other avian species is especially challenging, where the egg size is different from the chicken. In different experiments researchers used different specific criteria for selection of surrogate shells (27-30 g heavier than donor eggs, double-yolked eggs of a commercial broiler strain [288], suitable recipient chicken egg shell [34], 30 g heavier [222], 27-30 g heavier [255], bigger bed shells prepared for quail embryos from small chicken eggs by removal of their blunt-end halves [239, 240], cutting narrow end of the chicken egg at 35 mm diameter for quail [241]). Because of such specific criteria, it was rather difficult to select surrogate shell with different avian species laying eggs of different sizes, or even smaller or larger eggs of the same species. It was an important question to be answered whether the same criteria is applicable for all sizes of eggs or not, since avian eggs vary a lot in size and shape; like ≈0.5 g for Hummingbird and ≈1.5 kg for Ostrich (*Struthio camelus*) and shapes also vary from the nearly spherical eggs of owls to the sharply pointed eggs of murres and gulls [248].

The oxygen available to the avian embryo during development depends on diffusion across the eggshell and shell membranes, hence on the oxygen conductance (Go_2) of these structures. Eggshell conductance represents an important limitation to availability of oxygen in the embryo [204, 209, 211]. If embryos of different avian species had the same oxygen requirements (Vo_2/kg), the availability of oxygen would be more limited in the embryos of the larger eggs (usually laid by larger birds), since the surface

area/egg weight and Go_2/egg weight ratios are lower than in smaller eggs [16, 248]. However, interspecies analysis reveals that this is not the case. The Vo_2 of the avian embryo matches the structure and the Go_2 of the eggshell in such a way that the O_2, CO_2 and water vapour pressure difference across the shell are the same for a wide range of species, from the few gram eggs of the sparrows and quails to the 1.5 kg egg of the ostrich, implying a constant arterial oxygen tension among species [16, 133]. If the embryo's metabolic rate was a species characteristic, possibly genetically determined, then the mass of the egg may have some effects on the development of the embryo due to differences in oxygen availability and O_2 availability and Vo_2 are limited in the avian embryo incubated in 21% O_2, and that the conductance of the eggshell represents the limiting structure. *Intra*-species differences in egg mass and the embryo's Vo_2 is not an invariable species characteristic, but a variable dependent on O_2 availability. The adaptability of Vo_2 to O_2 availability is a property most likely related to the degree of functional development of the compensatory mechanisms against changes in the partial pressure of oxygen (Po_2). In the early stages of development the total O_2 requirements are very small, hence probably not limited by O_2 availability [392]. Limitation to O_2 availability becomes apparent at later stages of development, at which time the embryo's Vo_2 increases substantially (Figure 4.1). The permeation of air through the eggshell and the shell membrane is one parameter that affects the hatchability [353]. Since chicken eggshell is double in thickness than the quail and has different porosity which will influence the gas exchange across the shell and hence the embryonic growth and hatching.

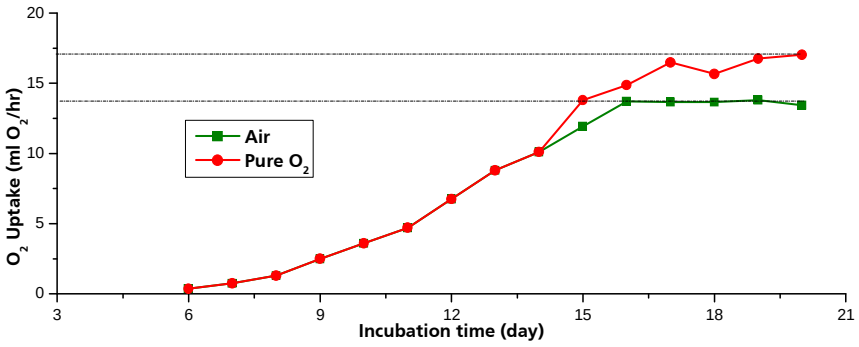

Figure 4.1: Oxygen uptake of bantam hen embryos incubated in normal air and pure Oxygen.
Redrawn after [129].

Assuming that an egg shell is a surface of revolution generated by revolving a curve y = f(x) about the x-axis (in this case the long axis of the egg), the surface area A is given by [248]

$$A = 2\pi \int_a^b y\left[1+\left(\frac{dy}{dx}\right)^2\right]^{1/2} dx \qquad \text{Equation 4.1}$$

where a and b are limits of integration as shown in Figure 4.2. If the functional relation y = f(x) is an analytical function, it may be possible to perform the integration directly. Since y as a function of x is not known in general, we resort to an approximation.

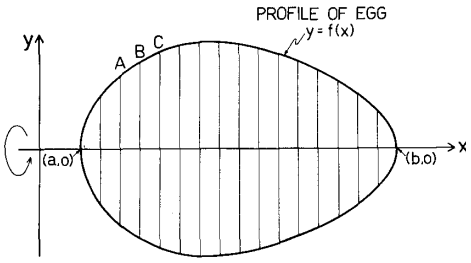

Figure 4.2: Profile of egg showing division into 16 segments. The direction of rotation is shown by the arrow [248].

Since there are a lot of natural variations among the eggs of different species, and even the egg laid by the same bird, an approximation will serve this purpose. Moreover, the measurement of the length and width of an egg is a tedious job, especially concerning the experiments conducted with hundreds of samples. Measurement of the weight would be rather easier. The relation between the surface area of the egg and weight can be calculated using following equation [248]

$$A = 4.835W^{0.662} \qquad \text{Equation 4.2}$$

Where **A** = **surface area of the egg** in cm^2, **W** = **weight of the egg** in g.
There is natural variability in egg shape and this variability can be characterized using a shape index (SI) [13] and sphericity (Φ) [9]

$$SI = \left(\frac{W}{L}\right) x\,100 \qquad \text{Equation 4.3}$$

Where **SI** = **Shape Index**, **W** = **width of the egg** in mm and **L** = **length in mm**

$$\Phi = \left[\frac{(LW^2)^{1/3}}{L}\right] x\,100 \qquad \text{Equation 4.4}$$

Where **Φ** = **sphericity (%)**, **L** = **length** and **W = width of the egg** in mm.

Since the CAM covers the whole inner surface of the egg and the eggshell is very thin and therefore negligible. The surface area of an egg can be considered as the approximation of the surface area of the CAM. Eggs can be characterized by the SI as sharp, normal (standard) and round if they have an SI value of <72, between 72 and 76, and >76, respectively [293]. Normal chicken eggs have an elliptical shape. Egg size and the eggshell thickness are strongly related to each other [116]. Eggshell quality depends on egg size and weight.

For all the experiments conducted in this thesis, eggs with shell index (SI) between 72 and 76 (normal/standard) were used. For selection of the surrogate shell, the ratio between the surface area of the donor egg and that of surrogate/recipient egg (SR) was used as an index, which was calculated using the following equation

$$SR = \left[\frac{\text{Surface (surrogate)}}{\text{Surface (donor)}}\right] X\,100 \qquad \text{Equation 4.5}$$

Where **SR** = **Shell Ratio**, **Surface (surrogate)** = **Surface area of the Surrogate shell**, and **Surface (donor)** = **Surface area of the Donor egg**; surface area was calculated using Equation 4.2.

Since different avian species have different incubation time, avian Embryonic Survival (ES) was calculated using following equation (%)

$$ES = \left[\frac{\text{Maximum survival (ID)}}{IP}\right] X\,100 \qquad \text{Equation 4.6}$$

Where **ES** = **avian Embryonic Survival** in %, **Maximum survival (ID)** = **Maximum survival of the avian embryo (Incubation Day)** and **IP** = **Incubation Period** of the regarding avian species till hatching in days. Here, ES = 100% means viable hatchlings at 21st day of ibcubation for chicken and 17th day for quail.

Table 4-1: Surface areas and volumes of eggs of different avian species [248].

Species	Weight (g ± SD)	Long axis (cm ± SD)	Area (cm^2 ± SD)	Volume (cm^3 ± SD)
Coturnix coturnix **(Japanese Quail)**	9.62 ± 0.66	3.09 ± 0.10	53.89 ± 2.13	8.80 ± 0.67
Gallus gallus domesticus **(Domestic Chicken)**	53.89 ± 2.13	5.67 ± 0.16	68.00 ± 8.51	50.95 ±11.40
Phasianus colchicus **(Ring-necked Pheasant)**	33.84 ± 2.34	4.61 ±0.19	48.05 ±2.23	30.81 ± 2.05

Preparation of the surrogate shell

The donor eggs are checked thoroughly for cracks, contour, shape and roughness of the shell. Eggs with cracked shell, rounded or slender shaped, irregular contour and rough irregular shell surfaces were discarded. Eggs were cleaned and disinfected with chlorine solution (Annexes). After drying in the air, they were stored in a refrigerator at 14 °C temperature. During experiment, the shell was again wiped with Bacillol anti-microbial solution and air-dried.

A circle of a suitable diameter was marked at the blunt end of the egg (containing the air cavity) with a pencil. The shell was than cut with a fine diamond cutter rotating disc (Proxon Engraving tool) leaving the shell membrane intact. The eggs were than held cut end downwards and rinsed with autoclaved distilled water to remove shell powder. Top of the shell was than removed with a scalpel and the content of the egg was drained out. Shells were again rinsed inside and outside with autoclaved distilled water. The prepared surrogate shells were than placed on an autoclaved tissue paper moisten with distilled water to avoid drought (Figure 4.3).

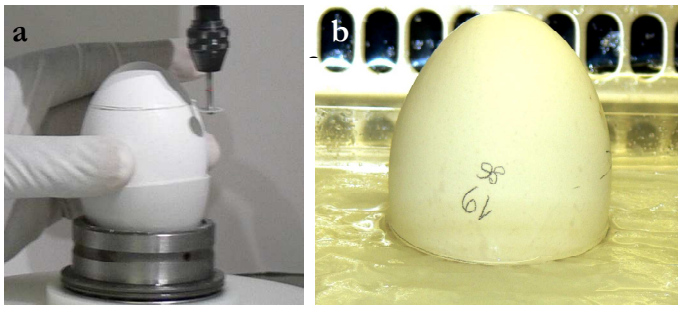

Figure 4.3: Preparation of the surrogate shell.

(a) Cutting the blunt end of the shell with diamond cutter; (b) prepared shell is placed on a wet paper towel to avoid drought.

4.5.1.3 Transfer of the preincubated avian embryos into the surrogate shells

Harvesting the donor embryos

For harvesting donor embryo, fertilized eggs from bantam chicken (*Gallus gallus domesticus*) or quail (*Coturnix coturnix*) were used. Bantam chicken lays relatively smaller eggs than the commercially available variety (White Leghorn or Rhode Island Red). Embryos are relatively stable on handling and have higher hatching rate than the commercial variety. On arrival, eggs were washed with water to clean and disinfected with hypochlorite solution (appendix), air-dried and sorted in a refrigerator at 14 °C for 24 hours. Eggs weighing 55±5 g for chicken and 14±1 g for quail were selected as donor for embryos. On the following day, they were placed in an incubator (Bruja Motorbrüter) at 37.5 °C temperature, 60% relative humidity and 90° rocking angle. chicken eggs were incubated for 72 hours and quail eggs were incubated for 48 hours. Eggs were rocked every half an hour.

The rest of the procedures were performed under a laminar flow cabinet adopting sterile techniques as far as possible. Figure 4.4 shows the complete procedure of avian culture in surrogate eggshell. Chicken eggs were taken out after 72 hours and quail eggs after 48 hours of preincubation, opened and transferred into the surrogate shell.

The trickiest part of the whole experiment was the transfer of the pre-incubated embryos into the surrogate shell. With the growth of the vitelline membrane following incubation, the egg yolk becomes very sensitive and vulnerable to shock, shake and movement especially during opening of the fertilized incubated eggs and shock from falling to the bottom of the container or even inside shell. Adequate care was taken during opening and pouring out to avoid any type damage from shaking or injury by the cracked shell spikes.

For harvesting preincubated embryo, incubated chicken eggs were opened with “Eggshell breaking and perforating device” (**Eierschalensollbruchstellenverursacher**) and quail eggs were opened with forceps (Figure 4.5). This method (applied only for chicken eggs) was found to be quick and easier than other methods described in the literature, especially for the fertilised incubated egg containing the growing embryo.

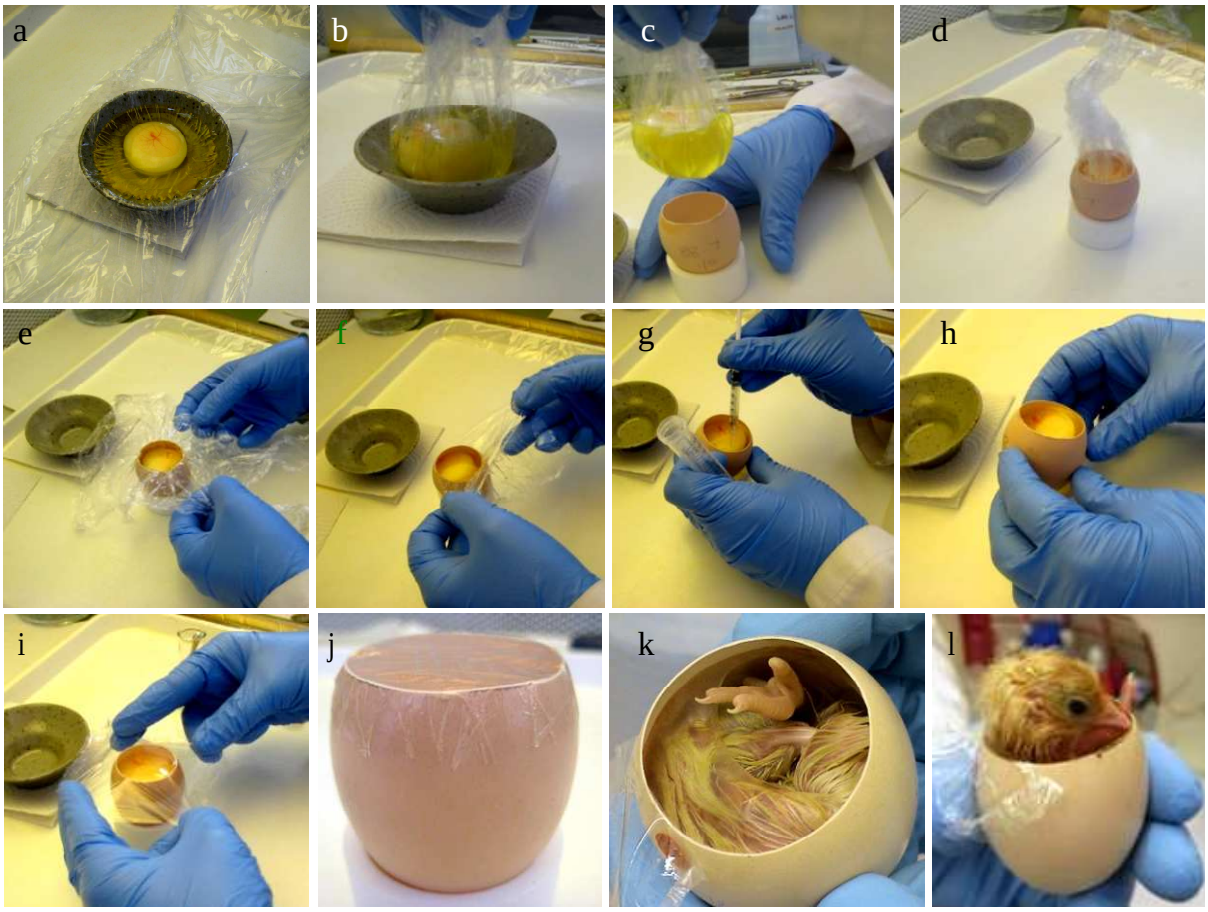

Figure 4.4: The complete schematics of cultivation of avian embryo in open system.

(a) 36 hrs old chicken embryo in cling film on a small dish; (b) Lifting the embryo by holding the four corners of the cling film; (c) pouring the embryo into the surrogate shell; (d) embryo along with the cling film inside the surrogate shell; (e) stretching the cling film in fan shaped manner; (f) taking out the cling film; (g) adding antibiotics; (h) rotating the shell for better mixing of antibiotics; (i) covering with cling film; (j) complete surrogate shell construct along with embryo; (k) internal pepping; (l) hatching (Imaging done at Roslin Institute, Midlothian, Scotland, UK).

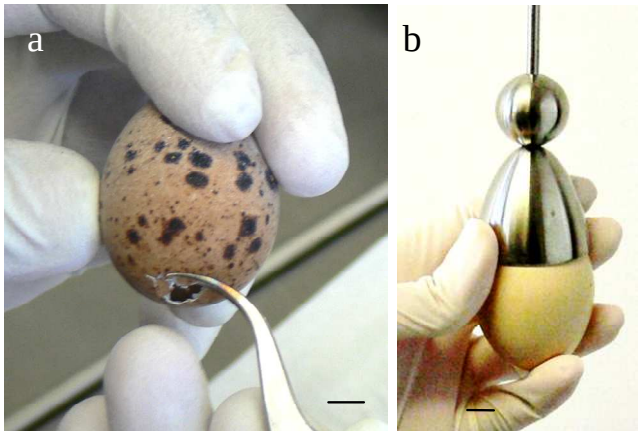

Figure 4.5: Methods for opening the donor egg for preincubated embryo.

(a) Quail eggs were opened with forceps, (b) chicken eggs with an "Eierschalensollbruchstellenverursacher" (Eggshell breaking and perforating device). Scale bar (a) = 5mm; (b) = 20 mm

Transfer of the harvested pre-incubated embryo into the surrogate shell

For transferring harvested pre-incubated embryos into the surrogate shells, either the method discrived by Rowlett and Simkiss [288] (Figure 4.4) was adopted or directly poured into the surrogate shell using a weighing boat. Normally the embryo floats at the top being the lightest of all. Otherwise, the yolk was turned with a spoon to bring the embryo to the top. Parts of broken shell were removed, if present. Following transfer of the preincubated embryo, 1 ml (for chicken, for quail 0.2 ml) of Penicillin and Streptomycin solution (P/S, Annexes) was added inside the surrogate shell taking care not to pour directly over the embryo. If necessary, little bit of thin albumin pre-warmed at 37 °C was added staying 8-10 mm belog the brim (3-5 mm for quail) of the surrogate shell.

Surrogate shells were painted with glue made from thin albumin (Annexes) on the outside wall of the shell from 5-10 mm from brim margin (3-5 mm for quail). A small piece of cling film was stretched with finger, placed over the open end of the surrogate shell, and wiped with a sterile paper towel. In the same way, a second layer was made over the first one. Excess part of the cling was trimmed.

4.5.1.4 Incubation of the avian embryos in the open system

The surrogate shell-embryo construct was incubated in an automatic incubated (Top-Profi 240) at 37.5 °C temperature, 60% relative humidity 30° side to side rocking (once every hour) for next 7 days. Than, rest 8 days with 60% humidity for chicken and 6 days for quail with 80% humidity without rocking. At day 18 of total incubation (for quail day 15), the embryo cultures were transferred to the Hatcher-incubator. The Hatcher temperature was maintained at 37-39ºC with high humidity provided by the evaporation of water located at the base of the unit. Embryos were checked for internal pepping (IP).

After incubation day (ID) 18, excess egg white was pipetted out to keep nostrils free to breath and prevent aspiration (ID 16 for quail). On the following days, after the establishment of pulmonary respiration, holes were made on the cling film cover to ensure air circulation according to breathing rate. Later at day 20, the covering cling films were loosened and if necessary, the beak of the cheek was freed from other body parts especially if it was covered by the wing and nostrils kept open for breathing.

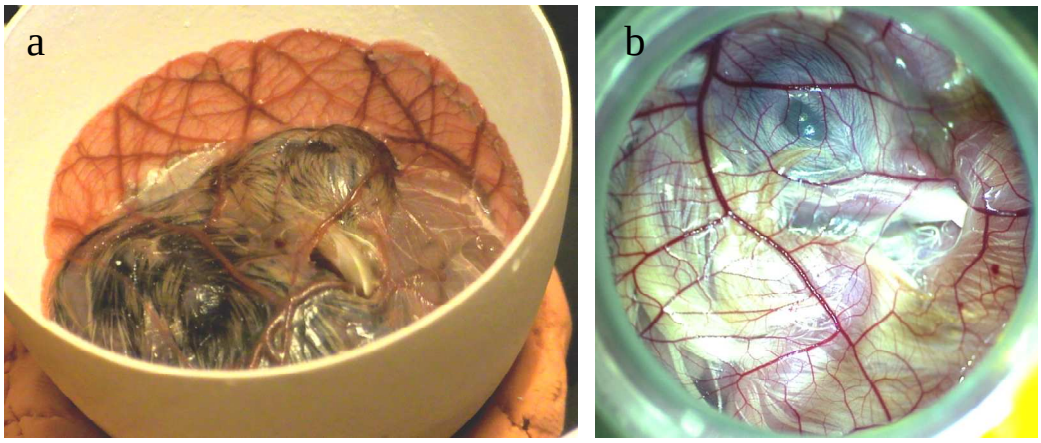

Figure 4.6: Avian embryos after internal pipping in open culture.

At this stage of development, the pulmonary respiration is established and the embryo is preparing for hatching (video supplied). (a) Quail embryo at ID 16 and (b) chicken embryo at ID 20 day in surrogate shell after internal pipping

If the embryos were dry, the under surface of CAM was moisten with distilled water soaked cotton bud. Normally the blood is withdrawn from the CAM circulation, and the embryo is ready to hatch. In normal cases, it came out of the shell through the loosened cling film at day 20 to 21 of incubation. In case of delay, embryos were manually removed and bleeding vessels were ligated with sterile threads. The chicks were than wiped with a dry tissue paper and returned to the Hatcher-incubator. Food (mashed poultry feed) and drink (fresh water with vitamin and mineral supplement) was provided on demand basis. For water, care was taken to avoid drowning of the hatchlings.

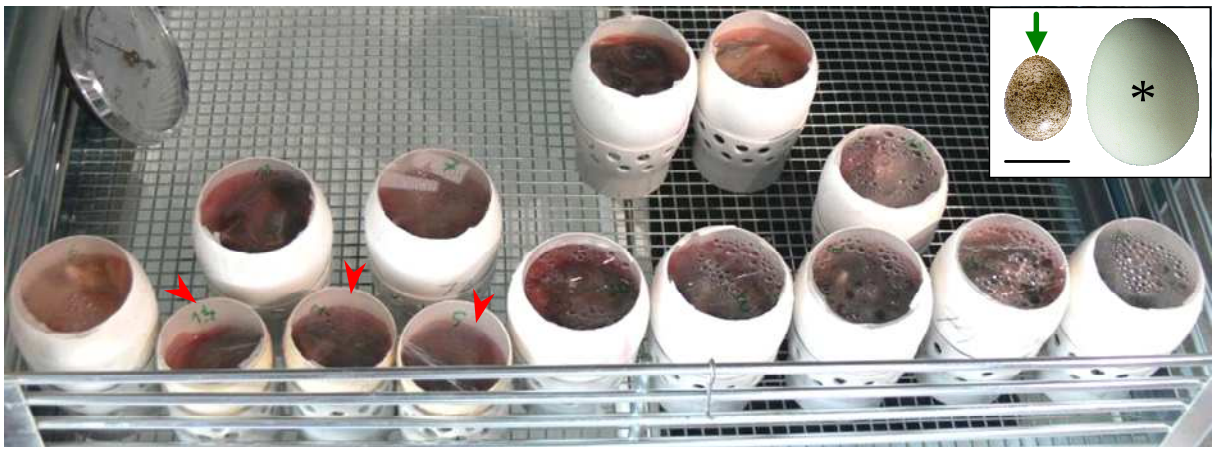

Figure 4.7: The open system of avian embryo culture consisting of surrogate shells inside a Hatcher-incubator.

Chicken and quail embryos after internal pipping. At this stage of embryonic development, embryos start breathing; holes were made on the cling film according to the breathing rate. (Red arrowheads indicate quail embryo in chicken shell). Inset shows a bantam chicken (asterisk) and a quail (green arrow) donor egg for comparison. Scale bar = 25 mm.

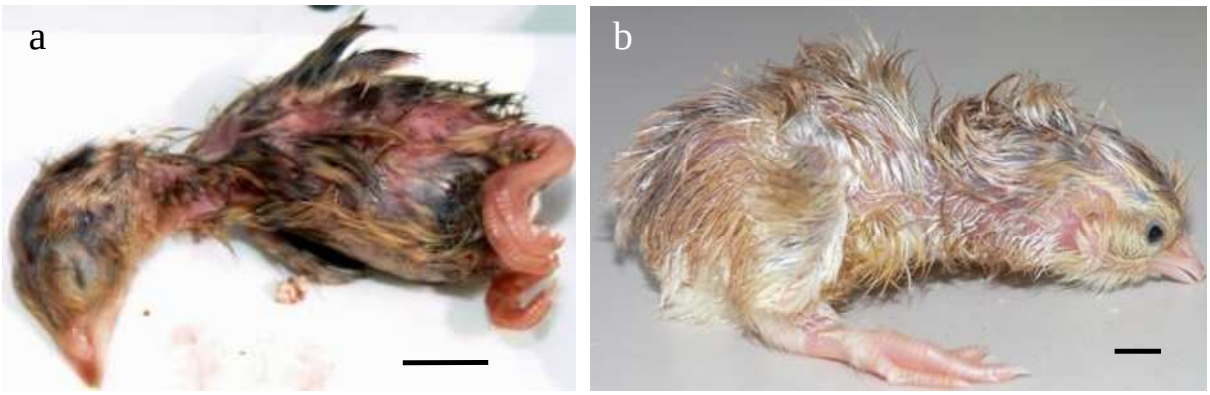

Figure 4.8: The hatchlings immediately after hatching form surrogate shell.

(a) Quail, (b) chicken. Scale bar = 5 mm.

4.6 The culture of the avian embryo in the completely artificial system

The complete artificial system for avian embryo culture contained no natural part of the egg except for the embryo with the egg yolk and egg white. The whole system consisted of a porous Teflon membrane (MilliWrap) suspended by a stainless steel (SS) net in a glass tube. The idea was to imitate the natural egg with complete artificial materials. The Teflon membrane replaced the egg membranes; SS gave the structural support to the embryo and the developing extra-embryonic membrane and calcium in organic form was added to the system for calcium supplementation for the growing embryo. In the natural eggs, the egg shell and the shell membrane protects against bacterial infiltration. In complete artificial system antibiotics were added to the system to prevent bacterial contamination.

When chicks were dry, fluffed and able to move around, they were transferred to a brooder box under a ceramic heat-lamp maintaining a temperature of around 35 °C. The hatchlings were obligatorily supplied with fresh water (with vitamin and mineral supplement) and food (mashed poultry feed) all the time.

4.6.1 The preparation of the completely artificial system

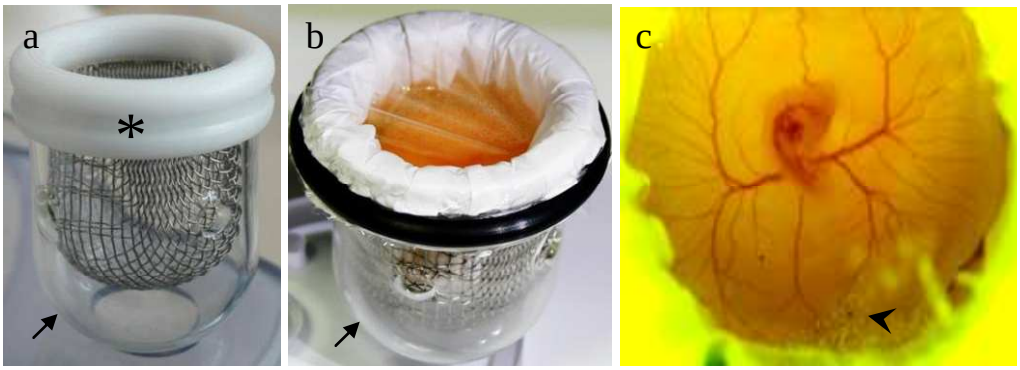

Figure 4.9: The complete artificial system of avian embryo culture.

(a) Complete artificial culture system; asterisk = plastic cover, arrow = glass container; (b) Open system containing 48 hours old quail embryo in Teflon membrane; (c) 96 hours old quail embryo in complete artificial system. Arrowhead indicates quail eggshell powder used for Ca^{2+} supplementation.

As mentioned earlier, the complete artificial system of avian culture was designed for culturing quail embryos. The system consisted of a glass container, stainless steel (SS) net (mesh width 1.4 mm, thread thickness 0.2 mm), a plastic cap and Teflon membrane. The glass container had an inner diameter of 30 mm, 45 mm high with diagonally placed four holes of 5 mm diameter in the middle circumferentially.

The SS net was pushed to form like a rounded bottom cup inside the glass container. The free end of the net was covered with a plastic cap. The whole system was autoclaved before use. 1 ml of distilled water was added inside the glass container at the beginning of the experiment. PTFE membrane (supplied as 5 cm width role, MilliWrap®, from Millipore Corporation; had 0.45 µm pore size) was cut into 50 mm square pieces and pushed into the artificial culture system with a rounded head pastel so that the Teflon lies on the SS net. Free ends of the Teflon membrane were trimmed.

4.6.2 Transfer and incubation of the preincubated quail embryos into the completely artificial system

The fertilized quail eggs were purchased from local farms. Sterilization was performed as before. Eggs were pre-incubatied for 48 hours in an automatic incubator at 37.5 °C temperature, 60% relative humidity and 90° rocking at 30 minutes cycle. 14±1 g eggs were chosen as donor for quail embryo. At the end of pre-incubation, eggs were opened with forceps very carefully and poured into a flexible weighing boat

(Figure 4.5). The embryos were inspected for developmental abnormalities. Parts of the broken shells were removed. Calcium lactate (Calcium L-lactate hydrate from Sigma-Aldrich GmbH, Germany) solution was prepared by suspending into the quail thin albumen at a concentration of 70 mg/ml. Quail eggshells were milled to form fine powder. 10mg quail shell powder, 25 mg calcium lactate or both were added to the artificial system prior to embryo transfer.

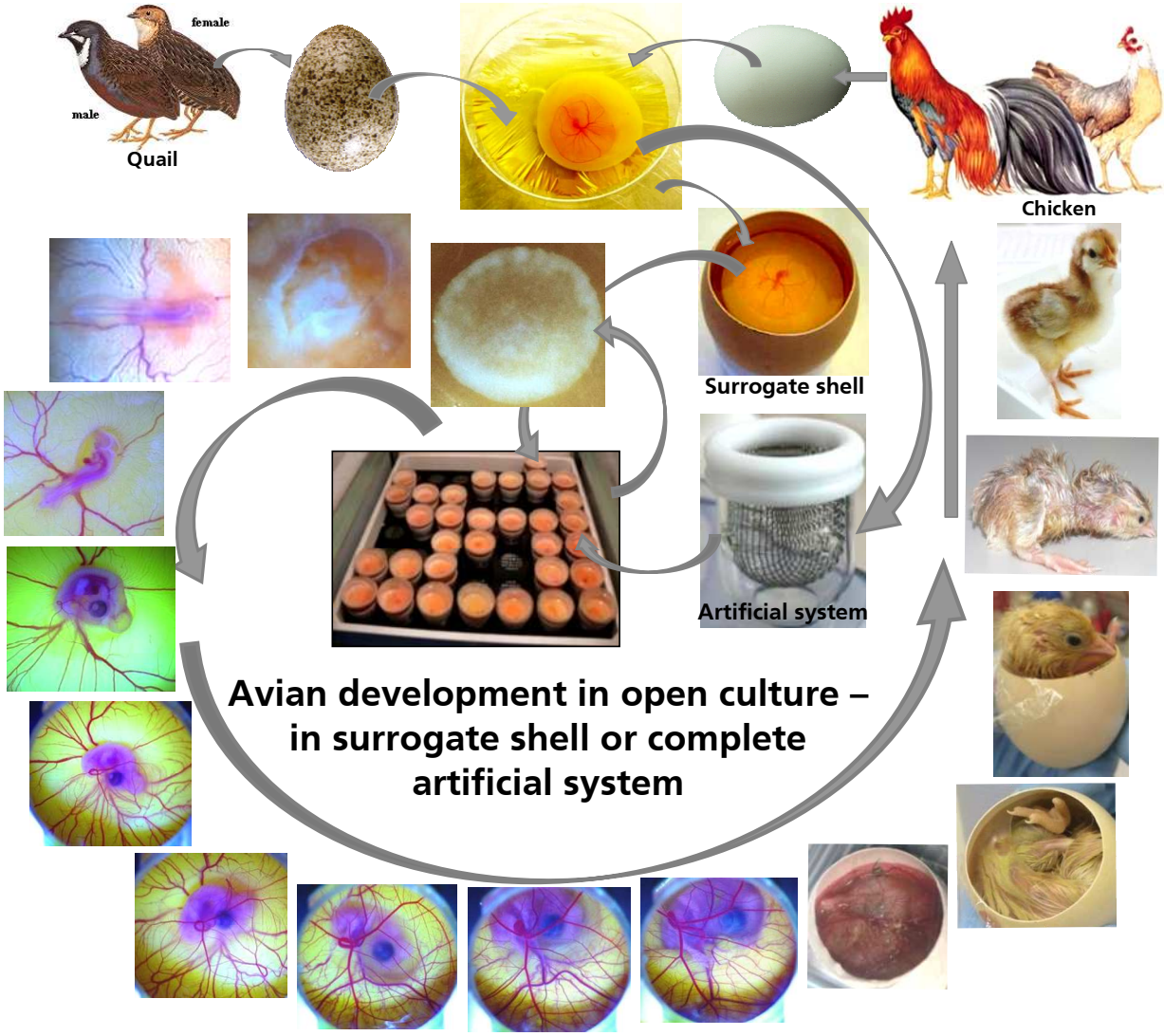

Figure 4.10: Overview of the open system of avian culture in surrogate shell or complete artificial system

The weighing boat containing the embryo was gently bent to form like a funnel and the embryo was slowly slipped into the artificial system. 0.2 ml antibiotics (P/S- see Annexes) were added to the culture at the side of the embryo avoiding pouring directly on to the embryo. The opening was closed with double layer of cling film and secured

with rubber ring. The whole system was than incubated at 37.5 °C temperature, 70% RH and 30° side to side rocking angle for next 10 days (day 13 of incubation). At day 14, rocking was stopped and the whole culture system was transferred to a Hatcher incubator with 60% RH and 37.5 °C temperature.

4.7 Results

Table 4-2 shows the summarized results of the culture of avian embryo in open system. It is important to note that the bantam chicken (SR = 133±5) had higher hatching rate (67 %) than white Leghorn breed (SR = 125±3 %, hatching rate 20%) even though they were cultured in the same system under similar culture conditions. Quail embryo cultured in bantam chicken surrogate shell (SR = 176±12 %) had only 14% hatching rate, whereas no quail embryo hatched when cultured in broiler surrogate shell (SR = 307±31). No quail embryo survived until hatching in completely artificial system.

Table 4-2: Summarized results of the culture of avian embryo in open system

Embryo	Incubation period	Surrogate shell	n	Hatching rate %	SR %	maximum viability	Comment
Bantam chicken	21 days	Broiler eggs	36	66.7	133±5	Hatch, ID 21	Higher hatching rate than broiler eggs, embryos are relatively stable on handling
White Leghorn chicken	21 days	Broiler eggs	15	20	125±3	Hatch, ID 21	Lower hatching rate than bantam chicken cultured under same conditions
Quail	17 days	Broiler eggs	62	0	307±31	ID 16	No one survived until hatching
		Bantam eggs	37	13.5	176±12	Hatch, ID 17	Very low hatching rate because of larger and thicker surrogate shell. 80% hatchlings had congenital malformation
		Artificial system	65	0	-	ID 8	No one survived until hatching, maximum survival was ID 8. Teflon is not a suitable material *in vitro* culture of avian embryo.

Figure 4.11 shows the graphical representation of the viability of different chicken embryos in broiler surrogate shell. It is important to note that only 20% of White Leghorn embryos managet to hatch in contrast to 67 % hatching rate for bantam chicken embryo, eventhough they cultured under similar conditions.

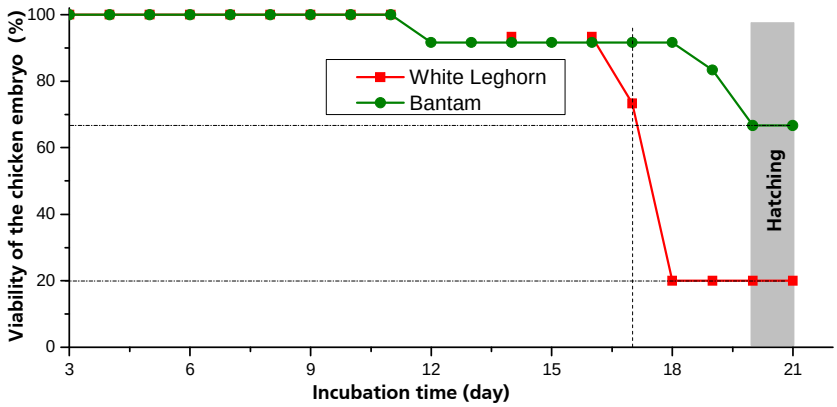

Figure 4.11: Viability of the chicken embryo in open system.

The open system consisted of surrogate shell and double layer of cling film. Note the hatchability was higher for bantam chicken embryos (≈66.66%) than White Leghorn variety (≈20%). After day 17 there was sharp decrease in the viability (≈16.66% for bantam and 53.33% for white leghorn). From the experiment it is quiet evident that the bantam chicken are more stable than the White Leghorn variety may be because the bantam is more wild breed than the White Leghorn which is genetically modified for commercial egg and meat production.

Figure 4.12 shows the graphical representation of the viability of quail embryo in different open system, which include surrogate shell from broiler and bantam chicken eggs, and complete artificial system. It is important to note that no embryo survived till hatching in broiler surrogate shell or complete artificial system consisting of Teflon membrane.

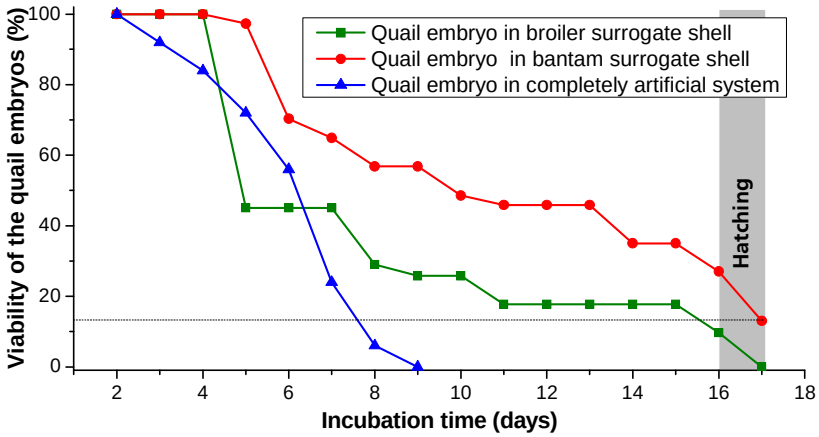

Figure 4.12: Viability of the quail embryo in open system.

In broiler surrogate shells, no quail embryo hatched, in bantam surrogate shell, 7% quail embryos hatched, in completely artificial system, no quail embryo hatched. Maximum survival of the quail embryos in completely artificial system were ID 8.

Figure 4.13 shows the graphical representation of ES (calculated by Equation 5) in relation to SR (calculated by Equation 4). It shows that the embryonic survival decreases with the increase of SR. This indicates that the embryonic viability is inversely

proportional to the surrogate shell size in open culture. Eventhough the graph is not linear, but gives an estimate about the inverse relationship. SR = 261 ± 8% in Figure 4.13 shows an upwords swing of the graph, which is probably caused by different surrogate shell used then the previous category (SR = 205.4 ± 4%).

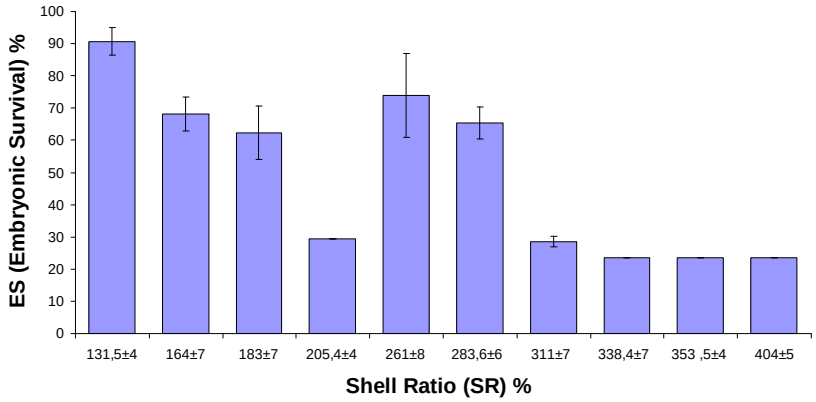

Figure 4.13: Effect of SR on the hatchability of avian embryo in open system.

SR was plotted against ES. From the graph, conclusion can be drawn about the inverse relation of SR with ES. Which literally means that embryonic survival decreases with increased of surrogate shell size. Here, influences other than SR were not considered. The graph is not linear because of different surrogate shell used for explantation culture may have different physical property that my have influenced embryonic growth and development.

Table 4-3 shows the health status of the hatchlings in poen culture system. Only 2.7 % chicks had notable congenital malformations in contrast to 80% in case of quails. In survival cases, SR was 133 % for bantam chicken than SR = 186 ± 6 % for quails. This result certainly gives a hint about the detrimental effect of larger surrogate shell in open culture system.

Table 4-3: Congenital malformations of the hatchlings

Embryo	SR (%)	% of malformations	Comment
Bantam chicken	133.4±0	2.7%	Abduction deformity of right leg
Quail	185.6 ±6	80%	Split leg deformity

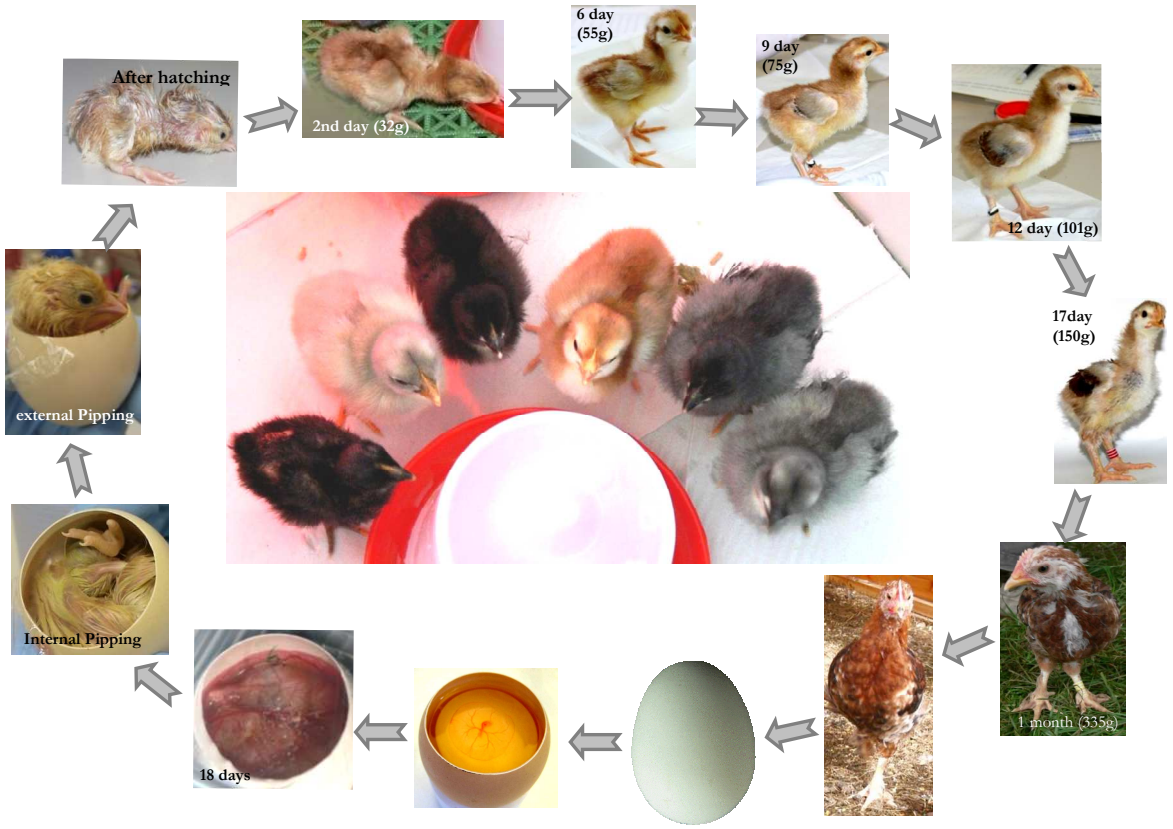

Figure 4.14: Culture of the chicken embryos in the open system.

4.8 Discussion

Culture of avian embryo is a magnificent way to study the avian embryogenesis realtime *in ovo*. Since it has a large opening covered with an optically transperant material, it might allow simultaneous observation and micromanipulation at the cellular level. However, it is necessary to identify different influential boundary conditions and factors to construct a stable system that can be further modified in future.

4.8.1 Effect of the surrogate shell size on embryonic survival

Figure 4.13 shows the relationship between SR and ES. Even though it was not possible to maintain similar culture conditions like SR and surrogate shell thickness similar to donor eggshell, but overall impression tells about an inverse relationship between hatching rate and SR; which literally means that surrogate shell has to be as close as possible to donor egg in surface area for having viable hatchlings. No embryo hatched when cultured in surrogate shell SR>190.

The architecture of the avian egg is of fundamental importance to the successful development of the embryo". Thus, "not only would manipulating of the ovum within

the calcareous shell be difficult" but if, as an alternative, a newly released ovum was used it was unlikely "that the remaining phases of egg formation—albumen and shell formation—would be achieved in the same or surrogate hen [97]. The problems with *in vitro* culture can be related to two main deficiencies. Firstly, the embryo is restricted both by the shape of the receptacle in which it is cultured and by the spatial constraints on the formation of the extraembryonic membranes. As a result, there is incomplete absorption of the egg albumen. Because it is a major source of water, electrolytes and protein for the embryo, it is not surprising that the embryos are retarded. Secondly, since the cultures are shell-less and since the eggshell normally supplies 80% of the embryo's calcium requirements, the embryos are hypocalcaemic and bone mineralization is grossly retarded. In fact the CAM, which normally transports calcium from the eggshell into the embryo [61] does not develop functionally unless the shell membranes are adjacent to it [73, 344]. The other abnormality of the shell-less culture is the absence of support for the embryo by the culture vessel.

The embryo on the yolk surface is fixed in an egg at the right position with two chalazae and may turn about inside its eggshell according to the gravitation force. It acquires the natural position by itself, what is necessary for formation of area pelllucida and body axis. Therefore, it is important not to disturb its natural position at the early stages.

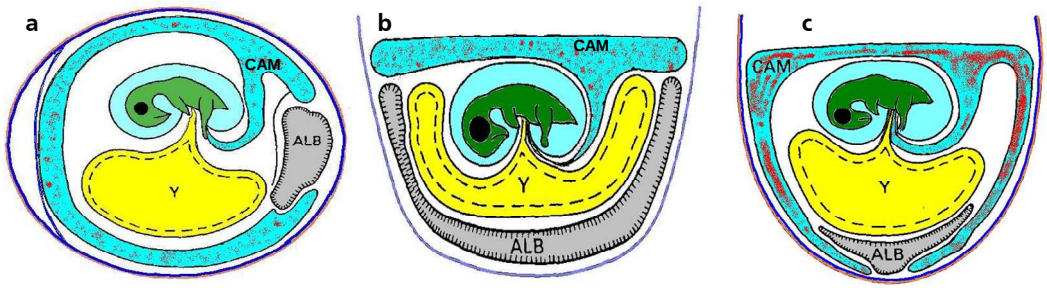

Figure 4.15: Arrangement of embryo and extra embryonic membranes in normal egg and different explantation culture.

Growth of CAM (a) in a normal egg (b) and surrogate eggshell (c) shell-less culture. Note the abnormal position of the embryo in the yolk and the superficial development of the CAM in the shell-less culture. ALB = albumen; CAM = ChorioAllantoic Membrane; Y = yolk (Modified after Rowlett et al, 1987 [288]).

By ID 4 allantois has fused with the chorion to form the CAM. This latter membrane continues to develop until at about ID 12 and it completely lines the inner surface of the inner shell membrane and is highly vascular. The chorioallantois becomes the major site for the exchange of respiratory gases until the embryo ventilates its lungs

during the hatching process [282]. The CAM surrounds the egg contents by ID 11 to 12 of incubation and fuses with the acellular ISM soon thereafter [57]. The ISM is bound to the overlying acellular OSM everywhere except in the air space region. Thus, *in ovo* the exogenous microenvironment of the developing CAM includes one or both shell membranes, a variable amount of Ca^{2+} and a steadily increasing concentration of CO_2 [378].

4.8.2 Effect of the surrogate shell shape and thickness on embryonic survival

As described earlier, for all the experiments conducted in this chapter, surrogate shell with shell index (SI) between 72 and 76 (normal /standard) were used for open system. It was not possible to maintain similar SR for all species of embryos (quail and chicken). For bantam chicken embryos, surrogate shells were selected from broiler eggs with SR 136 ± 5% (Equation 4-5) and the hatching rate was ≈67 % (Table 4-2).

Table 4-4: Shell thickness and pores of non-incubated eggs from several species of birds [16, 280].

Species	Egg weight (g)	Incubation time (day)	Shell pore volume (%)	H_2O loss (mg/day)	conductance mg H_2O/day.Torr	Pore length (µm)	Pores / egg	Shell thickness (mm)
Coturnix coturnix	9.60	17	0.47±0.06		14.40	170	3026	0.15±0.00
Gallus Gallus domesticus	60	21	1.47±0.21	343	3.10	350	12000	0.34±0.01

For quail embryos Instead, SR was 257 ± 68% and bantam chicken eggshells were used as surrogate shells- which has twice the thickness of quail eggshell (Table 4-4) and survival was only 5%. Bantam eggshells are different from quail in physical properties (Table 4-4). The egg yolk floats on to the egg white and blastoderm remains at the top being the lightest. If SR is higher, as was the case of quail embryo in large surrogate shell taken from chicken eggs, the egg yolk does not float on egg white, rather becomes flat. Figure 4.16 shows the computer simulation of quail embryo in chicken shell (b) and bantam embryo in Broiler eggshell (c). It was obvious from the experiment results that such case never led to hatching and nearly half of the embryos died in first week. In such cases of SR ≥ 191%, only 9 % quail embryos managed to survive until ID16. With SR 162-190 %, only 17 % quail embryos hatched. However, among the quail hatchlings, only 20% were normal; rest 80% embryos had congenital malformations- split leg deformity (Figure 4.17).

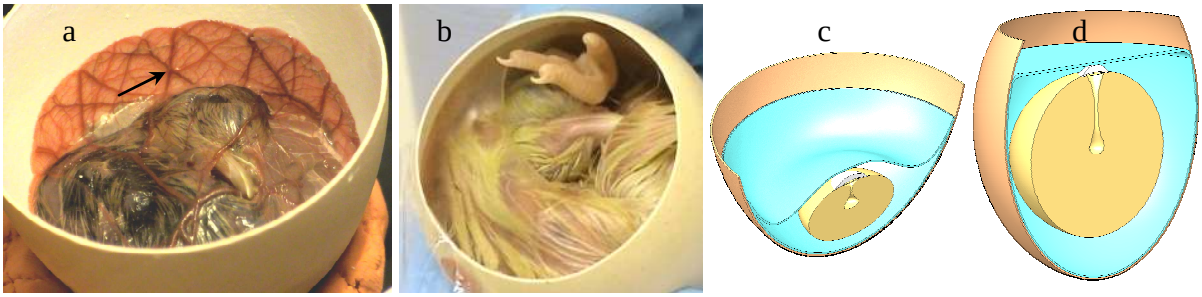

Figure 4.16: The effect of the large surrogate-shell on the developing embryo in open culture.

(a-b) Avian embryos after internal pipping: (a) quail embryo in a surrogate shell (SR ≈400%) at ID 16, (b) chicken embryo in a surrogate shell (SR ≈120%) at ID20. (c-d) Computer simulation of the effect of SR on open culture system. Note that the egg yolk is not round, rather became oval and flat in large surrogate shell (c) which was the case of quail embryo with surrogate shell from chicken egg (a). At later developmental stage, the embryo grows flat with incomplete development of CAM due to relatively large and flat surface. CAM formed double layer due to huge open surface which is evident by crisscross pattern (arrow) of CAM blood vessels in image (a). This was perhaps one of the reasons behind the maldevelopment of quail hatchlings in such system. This may have caused the incoherent growth of muscles and ligaments to the bone caused the maldeveloped limbs (Figure 4.17, Figure 4.18). (b, d) Chicken embryo in relatively smaller surrogate shell (SR ≈120%) grew normally with normal CAM formation and hatchlings were also normal.

This hatching rate and congenital malformation can not be generally in explained in terms of SR, since the culture conditions were not same for quail and chicken. However, the congenital malformation can be correlated with large SR (a-b in Figure 4.16). Normally the egg is full with the contents except for the blunt pole, and the embryo gradually attains the flexed posture following the curvature inside the eggshell (c-d in Figure 4.16). In open culture with large SR, the embryo develops relatively flat and legs are relatively extended. Perhaps the incoherent growth of muscles and ligaments in relation to bones is the reason behind such congenital malformations (Figure 4.17, Figure 4.18).

4.8.3 Congenital malformations: split leg deformity

As mentioned in section 4.8.2 of the current chapter, split leg type of congenital malformation were observed in the hatchlings where the SR was larger. In general, SR is an important factor that influence the hatching rate and the health of the hatchlings. Quail hatchlings born in such systems with large SR had split leg deformity, which is marked by the inability of the hatchlings to stand on the feet.

Figure 4.17: Congenital malformations, split legs.

Effect of too large surrogate shell on the embryo; hatchlings were born with congenital malformation of the legs (black arrow). They are not able to stand up on the feet. The left embryo is normal which was cultured in open system with SR 175%.

As described earlier, normally embryos develop in flexed posture tightly packed inside the shell. As the embryo grows, it gradually attains the flexed posture in its final position inside the curvature of the egg. This flex posture probably helps to develop the muscle and tendons coherently with the bone in the flexor and extensor surfaces. In surrogate shell with larger SR offers plenty of room for the developing embryo, which disturbs this flex posture (Figure 4.16) and perhaps the cause of incoherent growth of different groups of muscles and tendons that may have caused the deformity. Although this hypothesis could not be generalised because of different influential parameters in different experiments like different surrogate shell, SR, different species; but generally it can be concluded that if the SR is too large (SR>209), there were no hatching; if intermediate (209-183 %), the hatchlings had maldevelopment of both the legs (split leg, Figure 4.17). However, if the SR is somewhat smaller (133.4%), they had maldevelopment of single leg (Figure 4.18) and most often in the right leg.

The probable explanation for right leg deformity could be explained by the normal developmental posture of the avian embryo. Normally the head of the embryo is oriented towards the blunt pole containing the air cavity (in surrogate shell towards the opening) lies on its left (Figure 4.16). In general, if the SR of the surrogate shells lies in the border zone (155 %), the right leg develops relatively in extended posture; left leg remains flexed under the head (d in Figure 4.16) and is probably the cause of mal-development. In such cases, the malformation was treated by immobilization for for 24 hrs with spica (Figure 4.18).

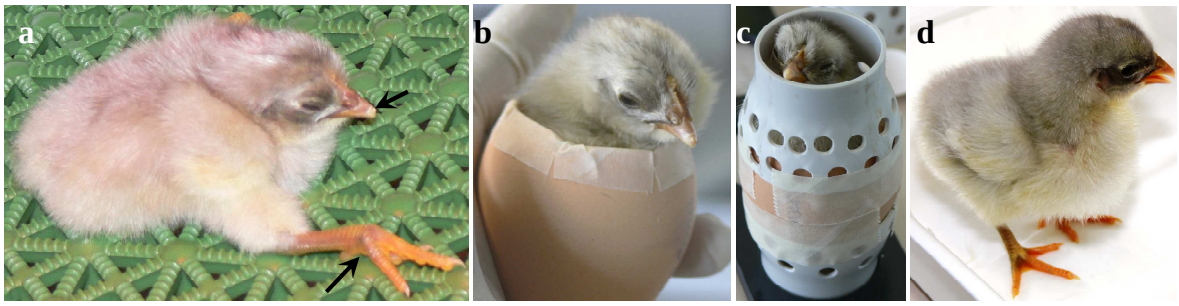

Figure 4.18: Congenital malformations (abduction deformity) of the limb and management.

In case of relatively smaller SR than in Figure 4.17, hatchlings had deformity in the right limb. Hatchling in image (a) had abduction deformity of the right lower limb. The deformity was treated with spica. The hatchling was put back into a larger shell for 24 hours for immobilization. (d) Same hatchling at day 5.

4.8.4 Congenital malformations: non-internalization of the yolk

Figure 4.19: Congenital malformation: non-internalization of the yolk.

(a) Hatchlings (White Leghorn) with non-internalised yolk; (b) management; (c) same chick one month post management.

Non-internalization of the remains of the egg yolk is one of the common congenital malformations encountered during the avian embryo culture in open system. The egg yolk supplies the nutrition for the developing embryo. With the onset of pulmonary respiration the remains of the egg yolk is drawn inside the body cavity as the embryo prepares for hatching after internal pipping (around ID19/20 for chicken, ID16/17 for quail). These remains of the egg yolk supplies the nutrition for the hatchlings for the first two days of life post-hatching. Precocial species learn to find food by that time (altricial hatchlings are fed by their parents).

In case, the yolk is non-internalized and the remains are small, it was pushed inside the abdomen using a sterile cotton bud adopting sterile techniques and secured in position by surgical adhesive tapes (Figure 4.19). If the external part of the non-internalised yolk is too large to push in, it can even be ligated using a sterile thread and removed after cutting with a sterile scissor taking care not to trap intestinal loops or large blood vessels in the ligature.

4.8.5 Absorption of Ca^{2+} from the shell

1-day-old chicks contain 26 mg of calcium, while both yolk and albumin contain only 6.4 mg. Therefore, during embryogenesis, it is estimated that this difference (19.5 mg of Ca^{2+}) is mobilized from the eggshell and accumulated in the embryo [80]. The CAM functions not only as a respiratory epithelium for the embryo [210] but also a mediator for asing of calcium from the eggshell and transport of calcium to the embryo for use in skeletogenesis [246, 247]. The transport of calcium involves a calcium-ATPase and a calcium binding protein and probably occurs via compartmentalization of calcium while it traverses the cytosol of capillary covering cells, the specific cell type that presumably is responsible for calcium transport [4, 5, 345-347].

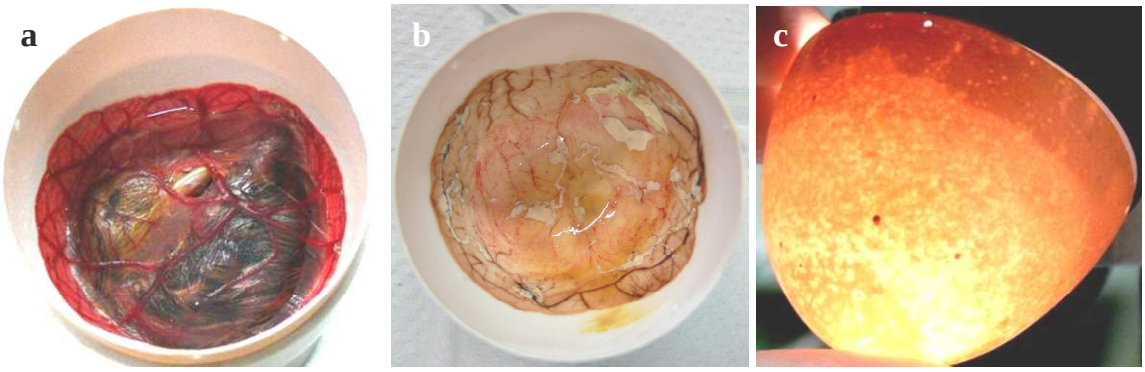

Figure 4.20: Calcium absorption from the surrogate shell in open system.

(a) A quail embryo with CAM after internal pipping (ID 15); (b) surrogate shell with extraembryonic membrane after removal of the dead embryo at ID 17; (c) trans-illumination imaging of the shell shows the area of the shell has become thin at the location of CAM due to Ca^{2+} mobilisation.

The CAM synthesizes receptors for the vitamin D hormone (1,25-dihydroxycholecalciferol or $1,25(OH)_2D_3$), and the hormone from precursors [59, 79, 80, 226, 228-230, 263]. Moreover, embryos (domestic fowl and Japanese quail) that are functionally deficient in $1,25(OH)_2D_3$ are hypocalcaemic, calcium deficient, rachitic, and do not hatch [77, 78, 80, 121, 122, 127, 228, 231, 232, 322]. This Calcium resorption from the eggshell makes it gradually thin and it plays an important role in water loss and gas exchange through the egg shell which match exactly with the growing O_2. As the eggshell becomes thinner gradually at the later stage of incubation, evaporation of water and gas exchange also increases gradually. This Ca^{2+} absorption can also be observed in surrogate eggshell where the shell underlying the CAM becomes thin (Figure 4.20).

4.8.6 Gas exchange through the eggshell

During the 21 days of incubation, the chicken embryo takes in ≈ 6 l of O_2 and eliminates 4.5 l of CO_2. For the majority of this time gas exchange takes place between the nest/incubator air and the blood capillaries in the CAM by diffusion across the shell and its associated shell membranes. O_2 consumption via the CAM increases exponentially as the embryo develops, but the diffusive process is inadequate to provide the energy required for hatching (Figure 4.1). The transition from diffusive gas transport via the CAM to convective transport via the lungs is accomplished within ≈24 hrs. O_2 consumption rises again when the chick chips the shell (external pipping) and continues to rise throughout hatching [43].

The increased embryonic demand for O_2 as incubation progresses leads to progressive changes in the blood. Although during the latter half of incubation the haemoglobin concentration in the red blood cells remains unchanged the total amount of haemoglobin in the blood increases due to an increase in the number of red cells. As a result, there is a change in oxygen capacity of the blood from about 8 cm^3/100 ml at ED 10, to about 13 cm^3/100 ml at ED 18 [329]. The oxygen affinity of the blood also increases due to falling concentrations of intra-erythrocytic ATP [214]. This results in CAM blood remaining 85-90% saturated with oxygen despite the decreasing air cell gas tension [329]. The barrier to gaseous exchange between the inner shell membrane and the capillary lumen also decreases because both the blood flow through the CAM and the capillary volume increase. The vascular bed of the CAM is supplied with blood by the allantoic artery, which forms a single stem as it leaves the embryo's body via the allantoic stalk. This stem divides into the right and left branches while passing on to the CAM.

4.8.7 Bacterial contamination in the open system

Bacterial contamination is one of the problems encountered during later half of incubation in the open culture system. The whole procedure was performed inside Laminar flow cabinet using all sterile materials adopting sterile procedures as far as possible. In addition, antibiotics (P/S) were also added to the culture system (1 ml for chicken ≈70 g donor egg, 0.2 ml for quail ≈14 g donor egg) during embryo transfer process. Even though, in many cases the culture system was contaminated with bacteria

from middle of the second week of incubation. The egg white appears milky in the contaminated culture (Figure 4.21).

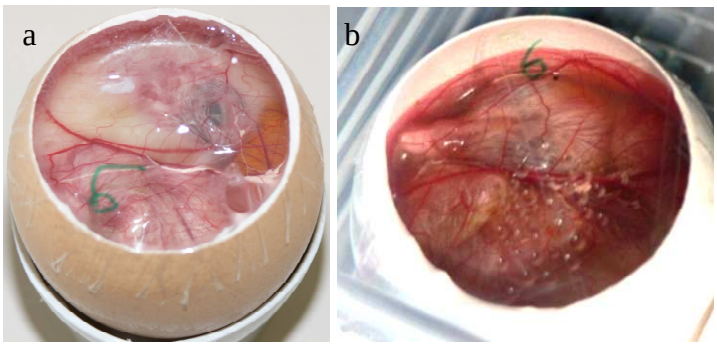

Figure 4.21: Bacterial contamination in the open system.

(a) Milky appearance of allantoic fluid of a chicken embryo in open culture at ID 16, (b) contaminated embryo after treatment with antibiotics at ID 18.

Though some embryos managed to hatch from the contaminated culture, most of them died before reaching the full maturity. One probable reason behind bacterial contamination could be the embryo transfer method using cling film descrived by Rowlett and Simkiss [288] (Figure 4.4). It was not possible to sterilize the cling film. Couple of turns of cling film from the role was discarded and the rest was used for experiments. Since the embryo along with the egg white comes in direct contact with the cling film during embryo transfer, the chance of contamination from the cling film is much higher. The quail embryo transfer was performed with sterile weighing boats, and there was no contamination in quail embryo culture.

In such contaminated cases, antibiotics (P/S) were added to the allantoic fluid. For chicken embryos, 1ml of P/S (0.2 ml for quail embryo) was taken in a sterile disposable syringe with a long needle and the needle was pushed into allantoic fluid through the double-layered cling film and the CAM avoiding large blood vessels. The antibiotics were injected underneath into allantoic fluid. The opening in the cling film was sealed with transparent adhesive tapes. Later the fluid became clear again indicating the functionality of the antibiotics (image b in Figure 4.21) and embryo managed to hatch in most of the cases.

Figure 4.22: 2 days old chicken hatchlings cultured in the surrogate shell explanation culture.

4.8.8 Egg turning

Most avian eggs need to be turned during incubation for normal embryonic development. The hatch rate decreases when eggs are not turned. Periodic turning of eggs is necessary for the complete achievement of normal development [400]. The unturned eggs showed regionally different decreases in the width of limiting membranes during embryonic development, it being delayed on the lower side of the eggs. Egg turning may affect other important factors involved in the production of chicks. Failure to turn eggs resulted in retarded development of the area vasculosa and extra-embryonic membranes, retarded embryonic growth, and reduced oxygen uptake and albumen absorption. Lack of egg turning results in poor chick hatching rate and delays the hatch for a few days. Reported explanations of increased mortality in unturned eggs are the embryos or extraembryonic membranes adhere prematurely to the shell membranes [236], the embryos fail to stimulate growth of the area vasculosa [66] or the embryos fail to line the entire interior of the shell membranes with the CAM. However, the hatch rate of unturned eggs with perfectly developed CAM is lower than that of turned eggs. The limiting membrane is made thin during the development over the whole surface with egg-turning, possibly through digestion of still unknown agents, and this thinning accelerates the rate of water permeation through the membrane.

The permeation of air through the eggshell and the shell membrane is another parameter that affects the hatchability [353]. The CAM structures are deeply engaged in calcium uptake from the egg shell, gas exchange, and egg-white resorption, thus sustaining the growth of the embryo and its normal hatch [55, 56, 75, 126, 227, 328, 345, 349, 350]. Ability of the CAM to spread around the inner surface of the shell membrane affects the growth of the embryo and that turning is especially important for embryonic development [354]. The thinning of the limiting membrane occurs in accordance with the development of the CAM and the thinning makes it easier for calcium and gases to enter or leave the CAM. Therefore, it is important that avian eggs should be turned for the limiting membrane of embryos to obtain enough thinned surface area to ensure a higher hatching rate [400].

4.8.9 Humidity and water loss from the egg

Water is important in the development of avian eggs that are laid on land. A certain amount of water must be around embryos to protect them from drying out at an early stage of development. Conversely, at a later stage of development, the drying of embryos is necessary for the embryos to initiate air breathing and adapt to living on land and to open the cloacal membrane [22, 267]. The eggshell and shell membrane may regulate evaporation of water from eggs. About 10 to 11% of the water is lost through these envelopes in domestic fowl eggs during the incubation period [354]. However, water vapor conductance of the eggshell (probably with shell membrane) is unrelated to the increased mortality of unturned eggs [354].

The main body of the shell membrane is made up of roughly parallel, intertwining fibers. The inner surface of the membrane is coated with a thin sub-layer called the limiting membrane or inner lining [128]. This layer is possibly composed of the same material as the cortex of the fibers [26] and may be important in directing the growth of the CAM during embryogenesis [42]. It may be the limiting membrane that retains the water and albumen because the spacing (8.0 µm × 37.5 µm) [25]; of the meshwork of the main body are large enough for water to pass through. During early stages of incubation, water might be kept inside the shell membrane by the limiting membrane in association with a colloid osmotic effect of the albumen.

Table 4-5: Water loss from avian eggs [282]

Fresh egg		Hatchling	
Mass (g)	Water content (%)	Mass (g)	Water content (%)
60	73.6	40	72.5

However, at later stages of incubation, it is absolutely necessary for water to move smoothly into and out of the space between the limiting membrane and the eggshell. About 80% of the calcium in the chick is obtained from the eggshell [143, 242, 305] and water is indispensable in the mobilization of calcium from the eggshell. Water permeability through the shell membrane increased at these stages in accordance with the decrease in the width of limiting membranes.

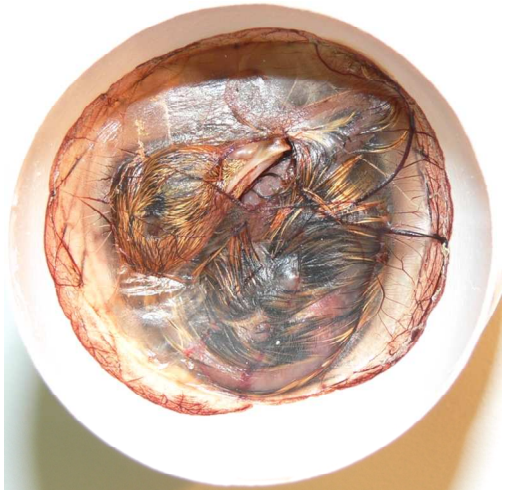

Figure 4.23: A dead quail embryo at ID 17 open system.

Note the embryo had internally pipped and dried in spite of 60% humidity inside the incubator.

However the incubation of quail embryos according to the incubation protocol used for chicken embryo in surrogate shell resulted in drought of the embryo (Figure 4.23). To prevent the drought, the humidity of the incubator was increased to 80% at ID14. Most of the scientific literatures in this regard are concerning chicken embryos and perhaps the water loss through the surrogate egg shell containing the quail embryo is different than chicken embryo.

4.9 Outlook

From a technical and biotechnical outlook, the eggshell is a sophisticated, highly developed, automated, dynamic culture system, which changes the boundary conditions permanently in accordance with the changing need of the developing embryo. *In vitro* culture of cells, in contrast is more static system where the boundary conditions are constant all the time; which is also reflected by the outcome of the system- cells divide and grow in a monolayer but remains as cells, much like microorganisms.

The zygote starts to develop soon after fertilization and as it comes down the oviduct, wrapped in egg white putting the cells at the liquid|liquid interface of two immiscible fluid- the egg white and egg yolk. Finally, the egg contents are packed with shell membrane and eggshell in CO_2 rich environment, which is essential for the cell in early days of embryogenesis. The cell division stops after the egg is laied due to cooling down. Even though porous, the egg is less permeable to gas at this early stage. It is able to maintain osmotic gradient at this stage even after drowning into water. With favourable temperature, the cell division resumes and the shell change its property gradually, synchronously and permanently with the growing demand of the growing embryo. At the end of incubation, the embryo consumes everything and strong enough to break the hard calcareous shell alone.

The experimental investigations in this chapter explored a spectacular method for observation of the vital processes during embryogenesis. The open system needs further modifications to make feasible for imaging methods, which will be emphasized in the next sections of this thesis. Technical modification of the open system consisting of bantam chicken embryo in broiler surrogate shell will enable to apply different imaging methods *in ovo* without interrupting the normal embryonic growth and development. Since optical imaging methods allow only imaging the optically transparent probes and only imaging at the surface, it is necessary to apply other imaging methods like µMRI in combination with long distance microscopes coupled with fibre optics to allow simultaneous imaging with optical microscope and µMRI.

5 Technical modifications of the open system for *in vivo* optical imaging and other methods

5.1 Summary

Objective: The overall all aim of the thesis was to make stepwise modification of the avian egg and develop a complete artificial egg system for *in vitro* culture of cells at the liquid | liquid interface imitating the same natural principle at the end. High resolution imaging play a very important role in studying the cellular behaviour *in vivo* to reveal the secret behind the organised cell migration, differentiation, tissue formation and formation of a complete viable organism at the end which is not possible *in vitro*. The main purpose of the experiments in this chapter was to make stepwise technical modifications of the open culture system and adapt for high-resolution optical imaging at the cellular level. Hatching of a viable bird was regarded as the parameter to asses the functionality of the modified system. Other methods of observations other then optical and characterization of the vital process of embryonic development were also considered; like recording of bioelectric signals and impedance measurement with the implantation of flexible electrode.

Methods: A combination of technical modification of the surrogate shell open culture system for better illumination and microscopy, construction of long distance fluorescence micro-imaging system, suitable cell manipulation system for *in ovo* application was developed and employed for addition/removal of cells to/from the culture system and monitoring them at cellular level. In addition, special flexible electrodes were constructed for *in ovo* application to measure the bioelectric signals from the developing embryo and characterise the embryonic development with impedance measurement.

Results: It was possible to make optical imaging of the developing chicken embryo from beginning of incubation until hatching with a very high resolution. The constructed micromanipulation system enabled injection of fluorescents labelled cells in the system at a desired location, which can be used for further experiments designed to follow the

fate of the injected fluorescence labelled cells during embryonic development without disturbing the embryo, which is normally done by histological methods.

Conclusions: The constructed system along with the modified surrogate shell open system can be used to study the organised cellular migration, cell differentiation, tissue formation non-invasively at least in the early stage of embryonic development when the embryo is relatively transparent. Application of such a system in the field of stem cell research, as well as for the embryology might provide a clue to the linage specific cell differentiation during embryogenesis. It may help to remove drawbacks of traditional cell culture methods, for better understanding of the process and the environment of the stem cell differentiation and tissue formation during embryogenesis *in vivo*. In addition, characterization of the whole process of embryonic development with impedance measurement with implanted flexible electrode array can bring additional valuable information to the existing field.

5.2 Materials required for the technical modifications of the open system

Equipments	Manufacturer
CCD Camera	Sony corporation, Japan
Filter	Edmund Optics, Karlsruhe
Thermocouple	Omega engineering, Deckenpfronn
Impedance analyzer	solatron Analytical, Farnborough, UK
Infrared camera	Jenoptik, Dresden
InfinityTubeTM stand in-Line assemblyTM	Infinity, USA
LED	Avago Technologies
Linear stage	OWIS, Germany
Manual micromanipulator	World precision instruments, USA
Rotary Measuring Stages	OWIS, Germany
Top-Profi 240 egg incubator	Hemel Brutgeräte, Germany
Ultra long working distance objective	Mitutoyo corporation, Japan
8 channels thermocouple input module	Omega engineering, Deckenpfronn

Chemicals	Manufacturer
Amphotericin B (250 µg/ml)	Fisher Scientific, USA
Bacillol AF disinfections solution	Bode Chemie, Hamburg
Calcium L-lactate hydrate	Sigma-Aldrich GmbH, Germany
Fluoresceindiacetate (FDA)	Invitrogen, Karlsruhe
Hypochloride solution (200-500 ppm)	VWR International GmbH
PBS	Invitrogen, Karlsruhe
P/S Penicillin/ Streptomycin	Invitrogen Corporation, USA
Silicone adhesive	NuSil Technology, USA
100% ethyl alcohol	Merck, Darmstadt

Biological agents	Manufacturer/supplier
Fertilised bantam chicken eggs	Anita Nefzger, Leutershausen
Fertilised White Leghorn chicken eggs	LSL Lohmann Tierzucht GmbH. Cuxhaven
Unfertilised broiler eggs	Glückliche Eier, Saarbrücken
L929 Mouse fibroblast cells	DSMZ, Braunschweig

Accessories	Manufacturer/supplier
Gold wire	Alfa Aesar GmbH, Karlsruhe
ITO glass	Präzisions Glas & Optik, Germany

Computer Software	Developer
IRBIS V2.2	Jenoptik, Dresden
LabVIEW	National Instruments, USA

5.3 Optimization of the open system for optical imaging

5.3.1 Background

Optical imaging is an extremely sensitive technique that can detect a single molecule using fluorescence techniques. It is usually performed in two modes: simple transmission absorption imaging and fluorescence imaging. In simple transmission absorption imaging, either transmitted or reflected light is used with tissue or optical probes, providing differential absorption to generate useful tissue contrast. Fluorescent imaging is performed by irradiating the tissue with a frequency of light higher than the emission fluorescence from intrinsic or extrinsic probes under investigation. Fluorescence imaging is the most sensitive approach, and it has gained great interest with the development of genetically encoded highly efficient fluorescent probes based on green fluorescence protein. The major limitation of light is the high absorption and scattering that occur in biological tissues and limit the penetration of the light through the body. Time-lapse microscopy has long been used to capture the dynamic nature of embryogenesis. Optically transparent embryos of animals such as *Caenorhabditis elegans* [321], sea urchin,[86, 110] and fish e.g. fundulus [342] or zebra fish [158], are well suited to studies of cell and tissue movement by time-lapse imaging using transmitted light (e.g. differential interference contrast [48] and various fluorescence microscopes. Fluorescent time-lapse microscopy has allowed the dynamic behaviours of labelled single cells or subpopulations of cells to be tracked *in vivo*. Of course, light cannot penetrate the hard shell, but can be applied in open systems. Since the embryo remains optically translucent in early stage of development, optical imaging with long distance objectives in conjunction with fluoresces can provide valuable information at or near cellular level.

Table 5-1: Effective permeability of cling film types used as covering materials in surrogate eggshell culture systems II and III [34]

		Effective permeability (a)		
	Thickness(µm)	O_2	CO_2	H_2O (b)
Saran Wrap	500	3.0	19.96	0.90
Handi Wrap	500	1600	7920	4.0

(a) Effective permeability = permeability [in mL/100 in (a) per 24 h/atm].

(b) Effective permeability to water vapour = permeability (in g/100 in (a) per 24 h/atm).

The open system of the avian embryo culture was originally constructed with chicken eggshell covered with double layer of cling film (from Lakeland, UK). The optical properties of the cling film is not very good for imaging. Furthermore, double layer of cling film make it even worse with trapped air in between the layers. Although the cling film is permeable to water vapour (Table 5-1), water condenses underneath the film cover giving the film foggy appearance which interrupts optical imaging. Gradually water droplets appear as the embryo grows. It was necessary to replace the cling film cover of the surrogate shell culture having materials with better optical properties. As discussed in Chapter 4, the explanation culture of chicken embryos in surrogate shell is an established routine procedure used for the production of transgenic birds. As the established system is functioning well, serving the purpose for transgenic bird poduction, there is not much attention to this issue. Since this method was designed for explantation culture, not for imaging and other types of experiments, scientific literatures are also not available in this field.

There are scientific literatures available regarding the permeability of the cling film to water vapor (Table 5-1). But an important question remained unanswered, "whether this permeability is necessary for the embryo survival or not". What happens if a material non-permeable to water vapor is used as a covering lid of the surrogate shell explantation culture? Because in the course of embryonic development, water is gradually evaporated from the egg and it is necessary for normal embryonic growth and development; especially at the later stage of the development, when CAM absorbs Ca^{2+} from the egg shell. Evaporation of water through the egg shell plays an important role at that time. All these issues were considered during the construction of the new covering lid for the surrogate shell.

5.3.2 Optimization of the covering lid for better optical imaging

The cling film cover was replaced with different material with better optical properties. This includes poly (methyl methacrylate) (PMMA) (Plexiglas), glass, and polycarbonate. Since the open system is rocked only to an angle of 30° during incubation, the upper part of the shell close to the opening is not exposed to the CAM. That was the reason behind the nonconsideration of biocompatibility of the cover material. After different trial, Plexiglas window with a groove filled with medical grade silicone was found to be feasible and finalized (Figure 5.1).

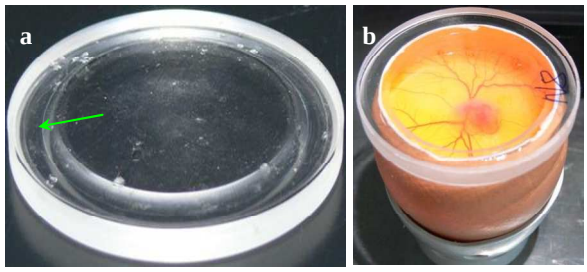

Figure 5.1: New lid constructions for the open avian embryo culture system.

(a) PMMA lid with silicone adhesive, (b) PMMA lid with silicone adhesive containing double glass window (black arrow) and tubing for warm air circulation (white arrow). (c) Chicken embryo at ID 5 in an open culture system with Plexiglas covers.

Plexiglas has very good optical transmission and it is suitable for optical imaging methods. Filling the groove on the side of the Plexiglas lid with a sticky medical grade silicone (NuSil Technology, USA) enabled placement of the lid without further preparations. It eased the whole embryo culture procedure where tedious process of placement, gluing, trimming and fixation of cling film could be avoided. The same cover could be reused after sterilization with 100% ethyl alcohol. After degradation, old silicone can be replaced with new one and is ready for use. It reduced the total time for the experiment and eased the process of preparation of large number of samples. Besides, both of the materials used in the construction of the lid are also biocompatible. The surrogate shell was opened with a fine diamond cutter and the new lid could be use as a cover like the cap of a glass container, which is easy to open and close.

Functionality and durability of the constructed lid was checked in terms of the hatchability of cultured chicken embryos. The chicken embryo explanted into a surrogate shell was closed with new Plexiglas lid and cultured until hatching (for shell measurement, look Chapter 4). The hatching of viable bird was regarded as the parameter to test the functionality and durability of the system. Figure 5.2 shows the results of the of the bantam chicken embryo cultured in the new system where ≈77% of the birds developed normally and came to the stage of hatching which was identical to the cling film system (67%).

These results indicate that the evaporation through the cling film had no significant effect on the hatching of the chicken embryo. The evaporation through the shell was sufficient to maintain the normal embryonic growth and development.

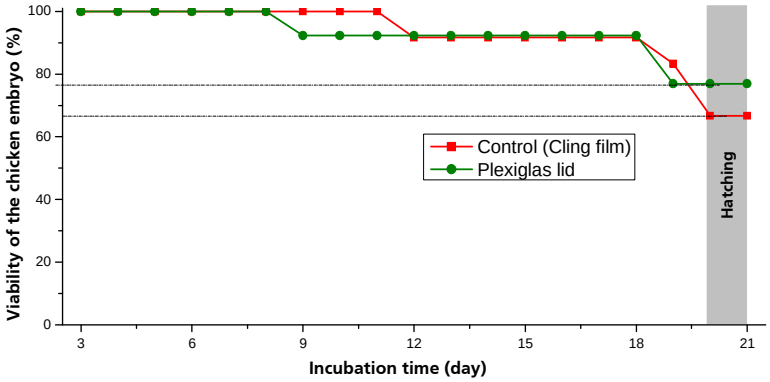

Figure 5.2: Viability of the chicken embryo in open system with Plexiglas lid and cling film.

≈77% of the embryos developed till hatching in open system with new constructed lid in contrast to 67% hatching rate with control. (N: Control =36, Plexiglas lid = 30).

5.3.2.1 Condensation underneath the covering lid

The open system was incubated inside a forced air incubator with active humidity control (Top-Profi 240). Chicken embryos develop their own mechanism of thermoregulation as they grow (they pass through a transitional stages from poikilothermy to homeothermy in precocial species of birds). In the early days of embryonic development they are not able maintain their own body temperature. Nevertheless, with the gradual development of thermoregulation, embryos develop their core body temperature, which is higher than the incubation temperature. An incubating bird uses the brood patch (a bare patch of skin that develops on the breast of many incubating birds) to keep its eggs at a temperature appropriate for incubation. Because an incubated egg is warmed only at one surface, it is rarely uniform in temperature. Temperature in the centre of the egg, or on the egg surface antipodal to the brood patch, are always cooler than the brood patch temperature (incubating bird sometimes use the brood patch to keep the egg cold in a very hot environment) [355]. The shell temperature (skin temperature) of the animal is at least 1-2 °C lower than core temperature [276] which means that the eggs are incubated at a lower temperature than the core temperature of the adult animal.

With the development of the embryo thermoregulation a thermal gradient develops across the optical window (inside warmer than outside) and therefore water vapour begins to condense at the inner surface of the window.

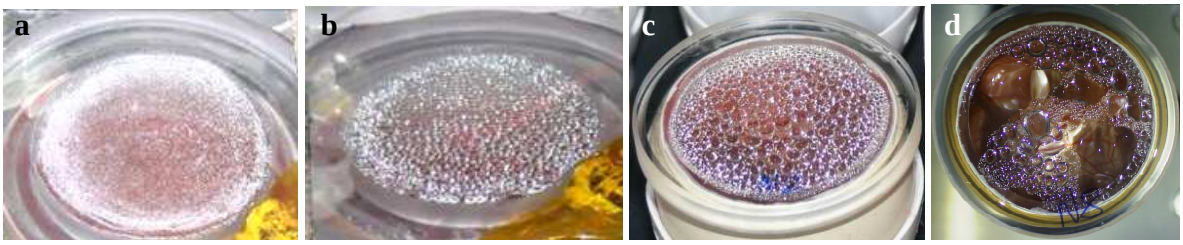

Figure 5.3: Gradual appearance of condensation with the growth of the quail embryo.

(a) ID 13, (b) ID 15, (c) ID 17, (d) ID 19. Note water droplet appears gradually with the development of the embryo.

5.3.3 Measurement of the thermal development of the chicken embryos

Although, the temperature of the egg content outside the embryo do not correspond to the actual core temperature of the developing embryo (at least in the later half of incubation), but thermal measurement of the fluid surrounding the embryo (egg white and later allantoic fluid) may give a clue to the thermal status of the developing embryo. In the incubator, the temperature is more homogenous. To investigate the reason behind condensation, thermal measurement of the developing chicken embryo was carried out.

5.3.3.1 Infrared thermography of the open system

Infrared thermography allows measuring the heat radiation from the surface. It is a very sensitive method to distinguish among the thermal radiation from the different point of a surface. Visible light cameras image in the 450–750 nm range of the radiation spectrum, Infrared cameras operate in wavelengths as long as 14 µm Instead.

The thermal imaging of the open system was performed to find out the thermal development. Thermal imaging was performed with a **"VARIOSCAN 3021-ST"** Infrared camera (from Jenoptik, Dresden, Germany). It had a Temperature resolution of ±0.03° measured @ 30 °C. Image analysis was performed with IRBIS V2.2 software provided with the system. Since thermographic cameras image the infrared radiation from the surface, thermal imaging of the developing embryo was not possible through the calcareous shell or the opening covered with lid.

Figure 5.4 shows the thermal image of the developing chicken embryos in open system at ID 15. In image (a), developing embryos show higher temperature than the control. In the zoomed image of the 15-day-old embryo in surrogate shell (b) and

control (c) shows the thermal difference between them and it is evident from the thermal image that living embryos have higher temperature than controls. During this experiment, the incubator door was opened, and time was allowed for thermal equilibrium. During this brief period, the control (unfertilised eggs) lost the heat but the embryos maintained. This indicates that at this stage of development, the embryos have developed their own thermoregulation.

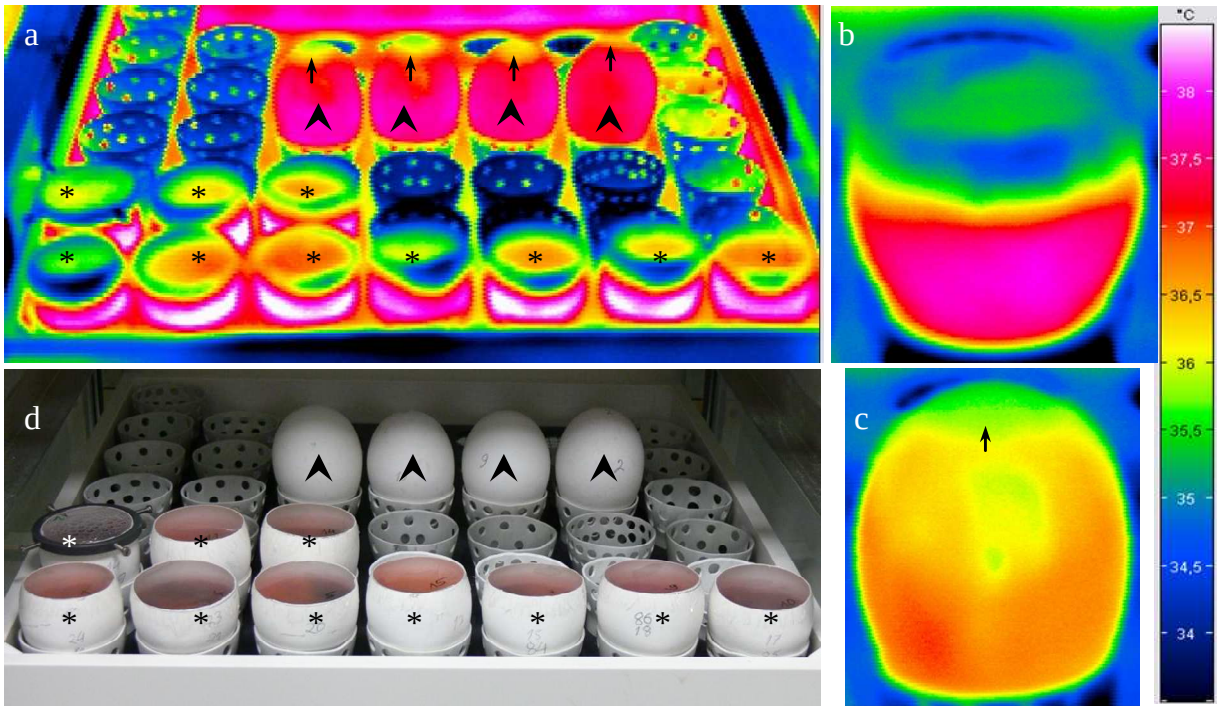

Figure 5.4: Thermal imaging of developing chicken embryos.

(a-c) Thermal imaging of the developing chicken embryo in open culture at ID 15 taken with a "VARIOSCAN 3021-ST" Infrared camera (from Jenoptik, Dresden, Germany). At this stage of development, embryos develop their own thermoregulation and they have a higher temperature than the incubation temperature. Four unfertilised eggs were place beside the growing embryos as control [black arrow head in image (a, d)]. Note in image (a), developing embryos show higher temperature [asterisks in image (a, d)] than the control (arrowhead). In thermal image of the control, the air cells (black arrow) are also visible as areas with low temperature. In the zoomed image of the developing embryo (b) and control (c) show that the developing embryo has at least 1 °C higher temperature than the control. In the thermal image of the embryo, the upper part of the shell shows lower temperature than the lower part because the CAM is not covering the inner surface of the surrogate shell completely, which can be seen in optical image (d) taken in the same position as the image (a) for comparison. All images were taken in the same scale.

To reveal the causebehind the condensation further, thermal development of the developing chicken embryo was carried out with thermocouple implantated inside fertilised chicken eggs.

5.3.3.2 Thermal measurement of the developing chicken embryos with thermocouple

Thermocouple wires were electrically insulated with a thin film of medical grade silicone (NuSil Technology, USA) and dried before the experiment. Butt-Welded Unsheathed Fine-Gauge Copper- Constantan thermocouple (Omega engineering) was connected to an "OMR-6018" 8 channels thermocouple input module (from Omega Engineering, Inc) and interfaced with the computer via RS-485 port. Custom written software in LabVIEW 8.5 was used for data acquisition (written by Dr. Robert Johan, "department of Ultrasound", Fraunhofer IBMT). Thermal measurements were plotted in a graph once every hour from the begining of incubation to external pipping (day 21 of incubation).

Implantation of the thermocouple into fertilised chicken eggs

Thermocouple implantation site was selected at the middle of the egg. Eggshell was ground off with a fine diamond-grinding tool leaving the shell membrane intact. The egg was rinsed with distilled water to remove grinded shell powder and wiped with a paper towel to dry. Previously prepared Silicone coated Fine-Gauge Copper-Constantan thermocouple was slowly implanted into the egg and surrogate shell. The opening was sealed with a small piece of sticky tape and the thermocouple implanted eggs were incubated further. The thermocouple was connected to the measuring system. Measurement was started nearly one hour later to allow the stabilization of the eggs in the incubator temperature. One thermocouple was placed inside the incubator for reference measurement. Figure 5.5 shows the image of experimental setup for thermal measurement with thermocouple.

Results of the thermal measurement of the developing chicken embryo measured with thermocouple

Figure 5.6 shows the results of the thermal measurement of the developing avian embryo during the whole period of avian embryogenesis measured with implanted thermocouple.

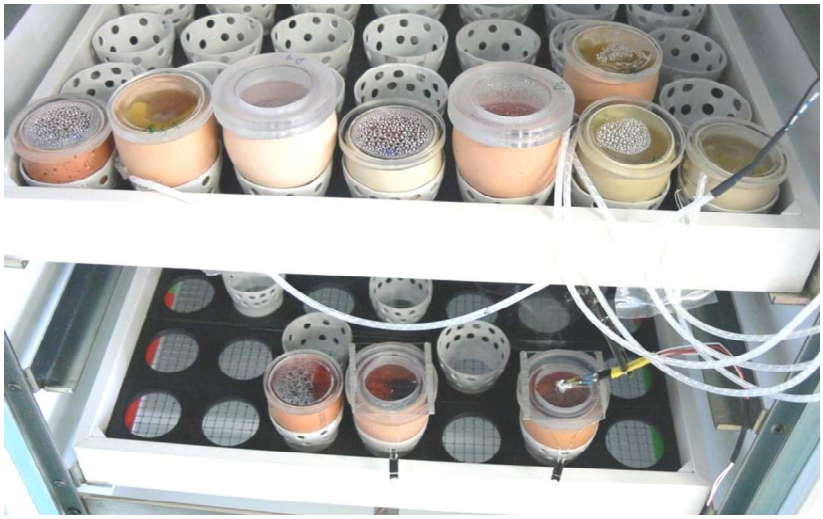

Figure 5.5: Experimental setup for the thermal measurement of developing chicken embryo with the sthermocouple.

Measurement of allantoic fluid temperature as an index of deep body temperature of developing chicken embryo. Copper-constantan thermocouple was placed inside the open system of the avian culture, which was in closed contact with the developing embryo

Note that the temperature of both developed and nondeveloped eggs remains nearly half a degree Celsius lower than the incubation temperature. Temperature of the developed egg starts to rise at around ID 12 and a thermal gradient of 1-2 °C develops gradually across the covering lid with colder outside than inside of the egg. This temperature gradient across the lid is the cause of water condensation underneath the lid. Temperature of the nondeveloped eggs remains the same throughout the whole period.

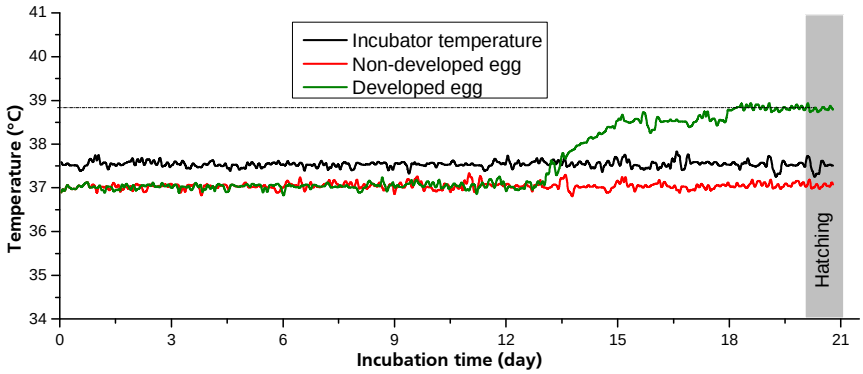

Figure 5.6: Thermal measurement of developing chicken embryo.

From the graph, it is clear that a difference in temperature develops between the embryo and the incubator in the second half of the embryonic development. Note the temperature rise of the developing embryo on ID 12.

With further growth of the embryo and persisting thermal gradients, condensation become intense and imaging becomes difficult. At a later stage, water droplets begin to appear. It was necessary to develop a special lid to remove the condensation quickly. This water vapour condensation interrupts imaging not only in the later stage of incubation, but also in the early days when opening the incubator door for short period create foggy condensation underneath the lid which takes long period to evaporate.

5.3.3.3 Removal of condensation: Resistive heating with Indium-Tin-Oxide (ITO) coated glass cover

In the early days of incubation, the condensation takes considerable time until it evaporates (nearly half an hour) when the temperature equalizes inside the incubator. However, for that latent period taken for evaporation of the condensed water, the imaging was not possible. In the later half of incubation, with persistent thermal gradient, condensation increases.

To solve the problem of condensation, different approach has been tried. Which include antistatic optical spray on Plexiglas window, Plexiglas ring with double glass plate on top and bottom, warm airflow between two glass plates, resistive heating with electro-conductive glass plate and others (Figure 5.7).

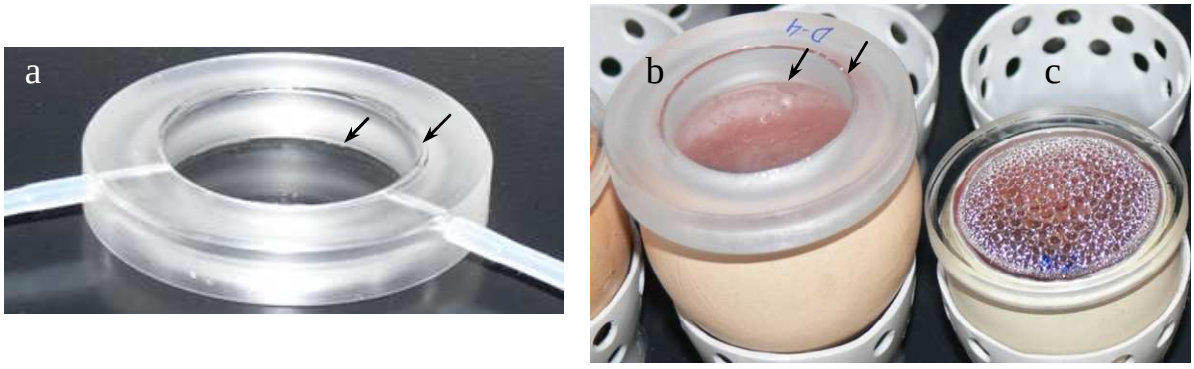

Figure 5.7: Different lid construction for the removal of condensation.

(a) Plexiglas lid with double glass window with possibility of warm air flow between the layers; (b) Plexiglas ring with double glass plate on top and bottom; (c) Plexiglas lid with silicone. Image (b) and (c) shows the effect of condensation under different types of lid construction at 14 day old embryo. Mark In image (b) (double glass window), condensation is less than normal Plexiglas lid (c), but not good enough for imaging.

Among all trials, warm airflow between double glass plates and resistive heating with electro-conductive glass plate found to be effective. Warm airflow requires extra set up, which is time consuming and with many samples together make the whole

procedure complicated. On the contrary, resistive heating with electro-conductive glass plate was effective and easy to use, quick to setup and temperature regulation is also simple.

ITO coated CEC020S glass was used as optical window (from Präzisions Glas & Optik GmbH, Germany). The glass was 1 mm thick, electrical resistance is 15 Ω/sq. and had 100 nm thick coating with ITO. It has very good optical transmission in visual region (≈90%). ITO glass was glued on a PMMA ring from the top. The window was heated with the flow of 20-40 mA DC current and the temperature was monitored with a Pt100 thermal sensor. Temperature was kept between 37.5 °C to 38 °C just to keep the lid window free of condensation (Figure 5.8). The upper limit of the heating was limited to 39 °C for a short period for avoiding thermal injury to the developing embryo. This method was very effective and found to be practical for long term imaging purpose.

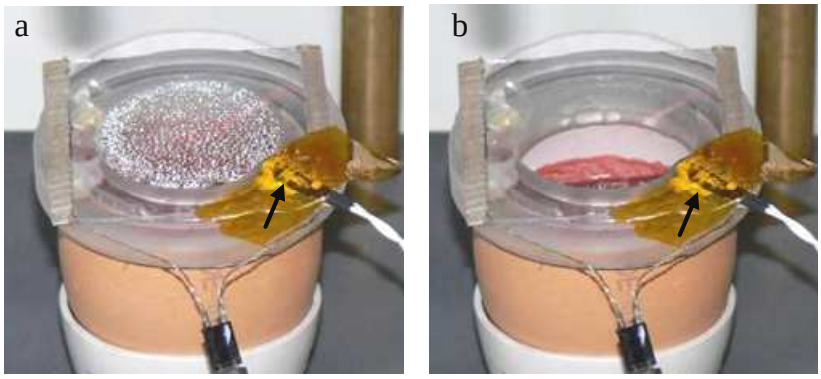

Figure 5.8: Removal of condensation: resistive heating.

chick embryo in open culture at ID18; (a) before and (b) after heating. Heating was accomplished with the flow of 20-40 mA DC current through ITO coated glass. The window temperature is monitored directly using a Pt100 thermal sensor mounted on the window (arrow).

5.4 Construction of a fluorescence microimaging and micro-manipulation system for manipulation and imaging of cells *in vivo*

The objective of this part of the current chapter was to construct a fluorescence micro-imaging and a micro-manipulation system for *in vivo* application that may allow the addition and removal of fluorescence labelled cells at the liquid | liquid interface of the chicken egg and track them *in vivo* in real time without the need of the histological preparation of the sample by secreficing the embryo. Since the embryo remains

relatively transparent in early days, the method can observe the cellular migration *in vivo* without disturbing the embryo.

5.4.1 Considerations for *in ovo* optical imaging

The open system is technically modified gradually and optimised for optical imaging. The yolk is the lightest part among the egg contents (specific gravity of egg yolk is 1.029, thick albumen 1.036 and thin albumen 1.040) [320]. The chemical composition of the thick and thin albumen is similar [64] but the thick albumen is richer in ovomucin which is responsible for the elevated viscosity [319]. Moreover, the specific gravity of thin albumen is lower than that of the thick albumen and also than that of egg yolk. If thin albumen is used, the ovum will float on the culture medium and the germinal disc may touch the upper lid. By contrast, if thick albumen is used, the ovum may sink into the medium and contact between the germinal disc and the cup that served as a lid can be avoided (Chapter 5). Contact of embryos with the solid plastic lid may harm their development. During these periods, the differences in the specific gravity among the yolk, thick albumen and thin albumen play a very important role in the normal development of chicken embryos [320].

Following embryo transfer, the surrogate shell was filled with thin albumen leaving nearly 10 mm free from the brim so that the embryo and the CAM do not touch the covering lid during rocking (Figure 5.9). For optimum imaging, good illumination is necessary; space is required between objective and the covering lid for illumination for reflection imaging.

Traditional optical imaging methods provide very high-resolution image. However, in most of the cases the short working distance of the microscope objectives makes it inapplicable for *in ovo* imaging. In addition, the eggs need to be rocked for optimum development of the embryo. Parallel cultivation of multiple embryos and observation at the same time requires an automated sample changing and rocking device. On one hand the whole system has to be robust and reliable, precise in movement in the range of micrometers for imaging at cellular level on the other. The imaging has to be performed in micro and macro scale: screening the whole embryo with macro lens to find out desired location for microimaging. Continuous illumination may have detrimental influence on embryonic development [326]. For long-term time lapse imaging,

it is therefore necessary to turn on the illumination only during imaging procedure and turn off afterwards. Traditional microscopes use incandescent or mercury-vapour lamps for illumination. Such lamps produce very intense light, which is than filtered to have desired frequency. However, they take quite a while to warm-up for emiting adequate intensity of light. It is very difficult to switch on and off such lamps within a short period.

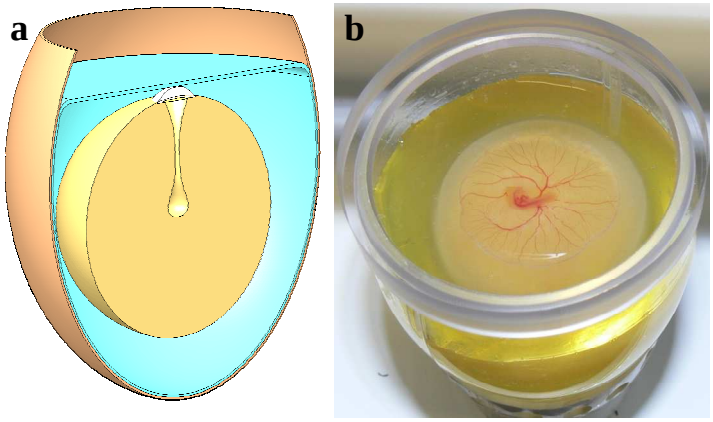

Figure 5.9: Position of the different contents of avian egg in open system.

(a) Computer simulation of egg contents (sectional view) shows the blastoderm floating at the top of the egg contents; (b) a 4-day-old chicken embryo in an artificial egg showing the developing embryo is floating at the top. Inside avian egg, the yolk is kept in position by chalazae, the yolk can rotate with the influence of gravitation, and the blastodermis uppermost being the lightest of all.

5.4.2 Construction of a long distance fluorescence micro-imaging system

It was difficult to adapt traditional microscopes for *in ovo* imaging. So for this especial purpose, an especial long distance fluorescence microscope was constructed with robust programmable microscope rocking stage with computer-controlled illumination for *in ovo* application. All processes were computer controlled and could be synchronised with each other.

Fluorescence micro-imaging system was constructed with an InfinityTube™ stand in-Line assembly™ standard series (from Infinity Photo-optical company, USA) with Mitutoyo 10×, 33.5 mm ultra long working distance objective (M Plan Apo objective 378-803-2 from Mitutoyo corporation, Japan) with 478-495 nm excitation Filter, 510-555 nm barrier filter (Blue excitation/Green emission) for green fluorescence imaging. Illumination module was made with nine pieces of "ASMT-JB31-NMP01" 3 W each mini power LED (from Avago Technologies). LEDs have dominant wavelength of 470 nm, Luminous Efficiency is 16 lm/W each. Nine LEDs were soldered on a 20 mm Bread Board

which was mounted on a round piece of aluminium with thermal paste in between for adequate heat transfer. A Sony "DFW-SX910" Colour Firewire CCD Camera was installed into the constructed micro-imaging system. The camera has an output image size (Horizontal x Vertical) of 1,280 x 960 pixel (SXGA) and it was interfaced with the computer via "IEEE 1394-1995" Digital interface.

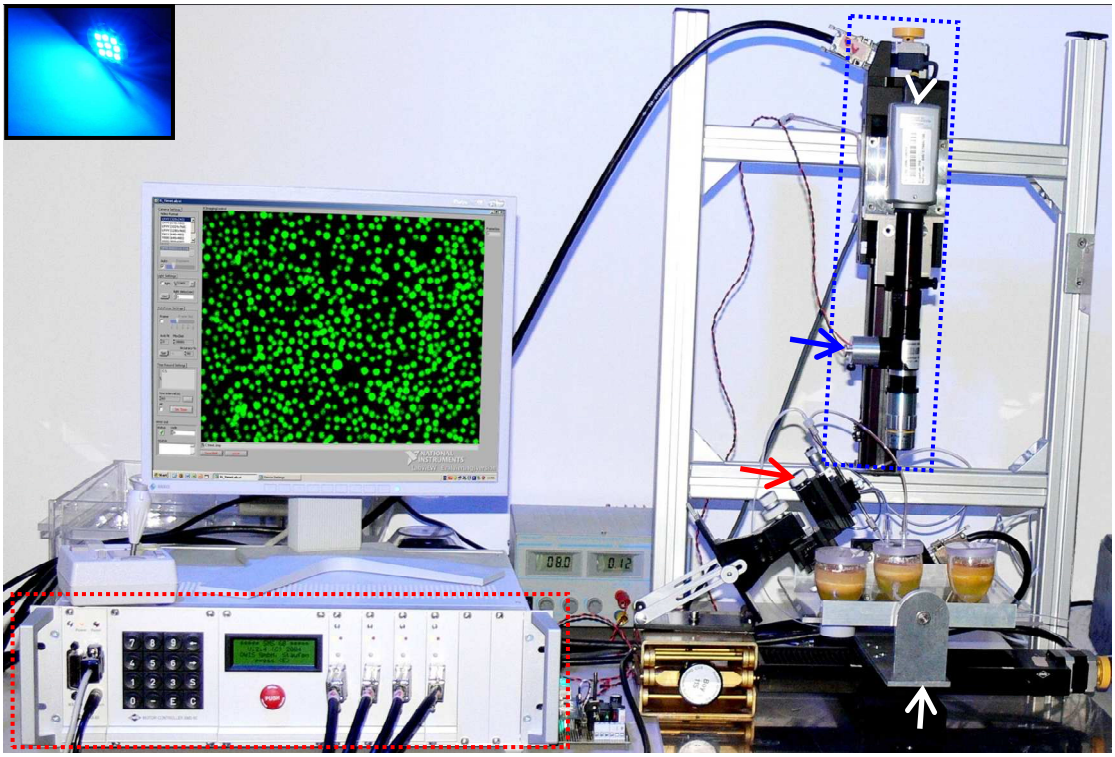

Figure 5.10: Fluorescence microimaging and micromanipulation system constructed for *in ovo* application.

Blue rectangle = constructed long working distance microscope mounted on high-precision linear stage; red rectangle = SMS 60 motor controller for controlling the microscope stage and camera auto focus; blue arrow = LED illumination; red arrow = micromanipulation system; white arrow = computer controlled XY stage for placement of culture systems for imaging; white arrowhead = camera mounted on the microscope; inset shows LED illumination module for fluorescence microscopy. In the computer display shows the L929 Mouse fibroblast cells (≈20µm size) stained with fluoresceindiacetate (FDA) imaged with the constructed system.

The XY Stage was made with two units of 41.091.036 C high-precision linear stage with 210 mm travel, 2-phase step motor and mechanical limit switches (OWIS GmbH, Germany) placed one over the other at 90° angle. Egg turning stage was made with a DMT 65 Rotary Measuring Stages (OWIS GmbH, Germany) placed on the XY stage. The Z-axis was consisted of another 41.091.036 C high-precision linear stage placed vertically on the XY stage. The camera was mounted on the Z-axis. A SMS 60 motor controller (OWIS GmbH, Germany) controlled the whole system. SMS 60 was

interfaced with computer via RS232 port. Custom written software in LabVIEW 7.1 was used for the stage control, camera auto focus and illumination (written by Leonora Petra, from "Ultrasound Department" of Fraunhofer IBMT). The advantage of the constructed system was all the processes could be synchronised with the custom written software and it was also possible to include necessary features on demand in the software to be controlled synchronously as required.

Results

The functionality of the constructed long distance fluorescence micro-imaging system was checked with imaging L929 Mouse fibroblast cells (≈20µm size) stained with fluoresceindiacetate (FDA) (Figure 5.10). Employing the new open system modified for optical imaging and the long distance fluorescence micro-imaging system, it was possible to image the whole period of the embryonic development of a chick in the form of time lapse imaging (Figure and movie 5.11). Still images taken from the time lapse video of the complete chicken development was placed in this figure for demonstration. It is important to note that the posture of the embryo and image contrast changed during the whole period of development due to extensive embryonic movement at later phase of development and resuscitation of the embryo during hatching. Note the embryo is relatively transparent during the early developmental phase that is extremely helpful for the tracking the fluorescence labelled cells injected into the embryo.

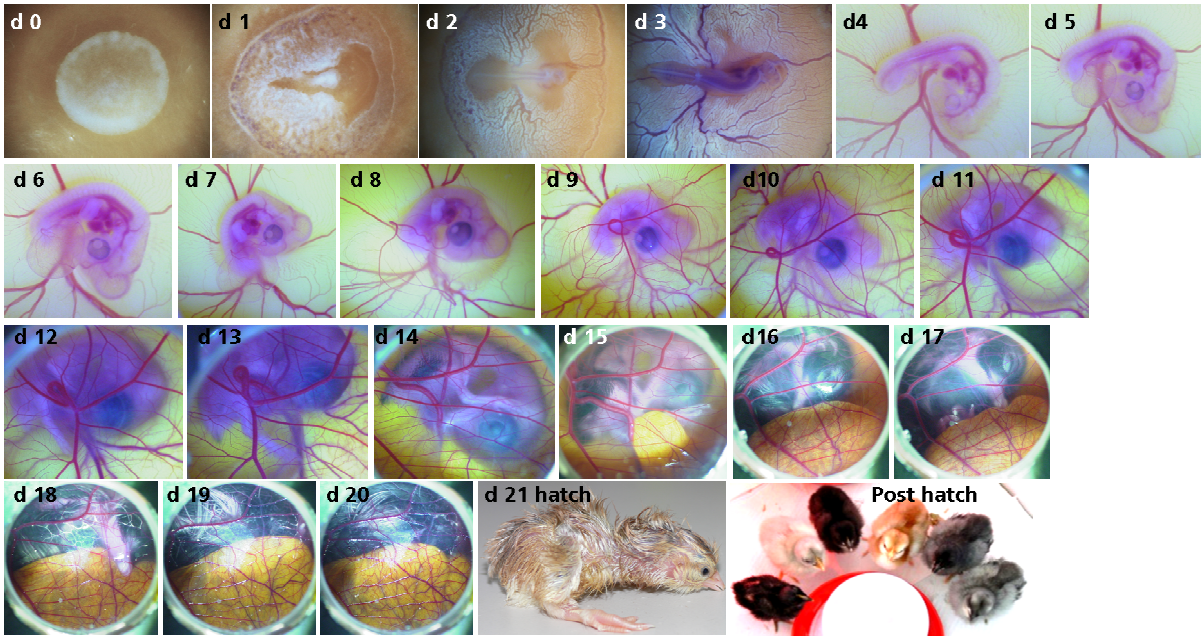

Figure and movie 5.11: Imaging of complete chicken embryo development in technically modified open culture system.

Still images taken from the time lapse video of the complete chicken development is placed in this figure for demonstration.

5.4.3 Construction of a micromanipulation system for *in ovo* application

Traditional micromanipulation system was modified for *in ovo* application. Micromanipulation system was constructed with a M3301-M3-R manual micromanipulator & tilting base (right-handed) (from World precision instruments) and a CellTram® vario system for microinjection and manipulation of cells (from Eppendorf, Germany) (Figure 5.12). The micromanipulator had vernier scales for readings to 0.1 mm & x-axis fine control for readings to 10 µm. It generates pressure with piston/cylinder system. It had a rotating knob for changing the volume, the volume change per revolution of the knob was 9.5 µl / 950 nl with total settable volume of 950 µl. The whole system was fitted with microcapillaries transfer tip (from Eppendorf, Germany). It was connected with a multiple channel valve regulator for attaching additional microsyringe so that the system could be used not only for addition of cells but also withdrawal of samples. This system was very convenient for injecting cells at a precised location or collecting small amount of samples. Since the whole system worked in conjunction with long distance microscope, precise manipulation was possible at cellular level.

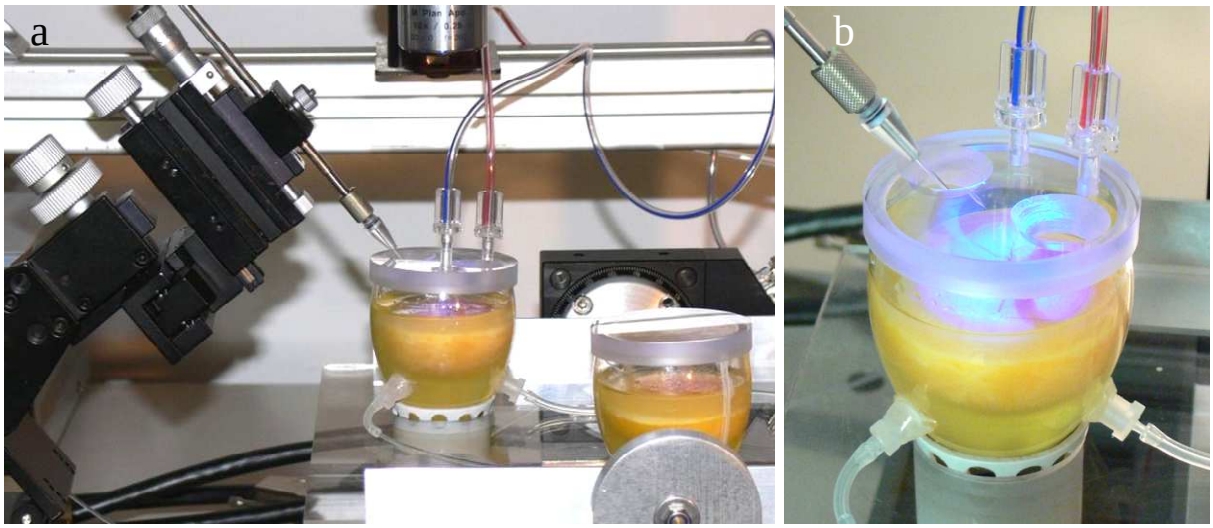

Figure 5.12: Micro-manipulation system constructed for *in ovo* application.

Micromanipulation system constructed for cell manipulation *in ovo*, for addition and removal of cells, injection/withdrawal of samples from the culture system. (a) Micromanipulation system in working position in an artificial egg; (b) zoom up view of the artificial system along with the microcapillary placed at the tip of the micromanipulation system.

5.5 Application of flexible electrode array for bio-electric signal acquisition and impedance measurement

The heart is one of the first organs to develop in the chicken embryo. The primordial heart begins to beat at around 30 hrs of incubation [282] which is also evident from the time lapse imaging of the chicken development (video supplied). In the early stage of development, the heart is very small. However, gradually, the primordial tubular heart forms the four-chamber configuration. The embryonic HR steadily increases, followed by relatively slow changes in HR during the late incubation period. Changes in heart rate (fH) during growth reflect the changing metabolic requirements and the state of the central nervous control of the organism. Functional vagal innervations appear on chicken embryonic heart on ID 12 [250, 251]. For that reason, measurement of Instantaneous heart rate (IHR), which is calculated from the beat-to-beat intervals of the heart and ECG could be an index of embryonic development and can provide additional information on physiological status and growth of the embryo. In addition, characterisation of the embryonic growth with impedance measurement could provide additional information.

Various methods and systems have been developed to detect cardiogenic signals through the eggshell. Those include electrocardiography (ECG), impedance-cardiography (ICG), ballistocardiography (BCG), acoustocardiography (ACG), catheterization of allantoic blood vessels and pulse oximetry. Each method has its

advantages and disadvantages and should be used individually depending upon the goal of investigations of embryonic HR.

Electrocardiography

The electrical activities of the heart in chick embryos within an eggshell were measured by Bogue as early as 1932 [31]. The electrocardiogram (ECG) was measured by a string galvanometer and the average HR was determined from day 1 to day 19 of incubation and after hatching.

Measurements of both ACG and arterial blood pressure become difficult toward the end of incubation due to augmented embryonic activities and respiratory movements, which disturb the ACG signal, and due to shrinkage of the allantoic artery, which makes it difficult to implant a catheter. Toward the end of incubation, the embryo pierces the air cell with its beak through the CAM and the inner shell membrane (internal pipping IP) and thereafter breaks the eggshell with its egg tooth (external pipping EP). During IP and EP period, which is defined as the perinatal (or paranatal) period, the embryo begins to breathe air and the gas exchange is switched from the CAM to the lungs. Both the BCG and the ACG can be detected sometimes even during the perinatal period when the embryos are quiescent, provided an adequate position for detection is found on the eggshell for placement of the transducers [330, 331, 333, 336]. However, neither method can detect the cardiogenic signals from early embryos because the BCG and ACG signals are weak or are not produced during the early incubation period. Alternatively, the ECG and ICG can be detected from early embryos, although the implantation of electrodes injure, albeit minutelyl [6, 131, 254].

Due to the availability of avian embryo especially the chicken and interests of the electro physiologists, there had been a lot of study on the electro-physiological measurement on avian embryo [44, 115, 180, 254, 259, 268, 334, 335, 332, 376]. However, most of the experiments were concerned regarding the studies during middle and end of the incubation. Since the primordial heart is very small at the beginning and the electrical signal amplitude is very low, it is not possible to measure such feeble electrical signals with the electrodes placed on the side of the eggshell. On the other hand, placement of conventional needle or other electrode with hard materials in early days of incubation in close proximity to the embryo will certainly injure the embryo and the egg yolk. In the later stage of development, physical movement of the embryo brings motion artefacts

into the ECG signal. Flexible electrodes constructed with biocompatible substrate placed closer to the embryo may ease the process.

5.5.1 Flexible polyimide-based electrode array for *in ovo* application

A special flexible and thin platinum microelectrode was constructed with biocompatible substrate to match the demand of electrophysiological measurement in the open system from beginning of incubation until hatching. The electrode material is platinum deposited on polyimide substrate and 10 µm thick. The whole electrode construct is 50 mm long and had 4 electrodes placed at the end with 1 mm diameter each 5 mm apart from each other. (Figure 5.13) Such electrodes are designed for implantation into the human body (Neuroprosthetics) for recording bioelectric signals from the human body or stimulation [49, 52, 162, 174, 182, 233, 234, 287, 315] .

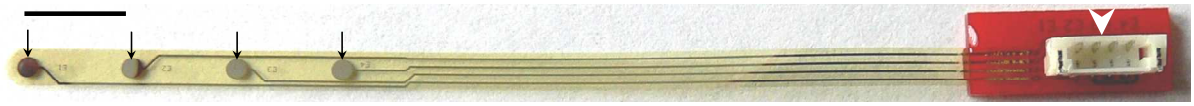

Figure 5.13: Flexible polyimide based electrode array for *in ovo* application.

Especially fabricated flexible polyimide based electrode array with four platinum electrodes electrode (thickness 10 µm) for recording bioelectric signal from developing chicken embryo and for impedance measurement. The electrodes are made of platinum (black arrow), exposed area 1 mm in diameter, and 5 mm apart from each other. Only the electrode surfaces are open, rest of the parts including the connections are insulated. The whole electrode is 50mm long. It has a connector jack (black arrowhead) to connect with bio-amplifier. Scale bar = 5 mm.

Flexible electrode structure was microfebricated by Thomas Dörge from the "department of Neuroprosthetics" of Fraunhofer IBMT in accordance with the need of the developing chicken embryo. For the fabrication of the microelectrode, the process technology was used as described [314, 316]. As substrate and insulation material, flexible Polyimide PYRALIN Pl 2611 (Du Pont) was used since it has a low water absorption and low conductivity [67, 82]. Platinum was used for the electrode material due to its biocompatibility [338] and electrochemical stability [294].

5.5.2 Measurement of the electrical characteristics of the flexible electrode array

The impedance characteristic of the electrode structure in two-electrode and four-electrode configuration was determined experimentally in electrolytes with known electrical properties. The impedance spectrum of the fabricated electrode array was

calculated using an impedance analyzer and interface (solatron Analytical, Farnborough, UK). From the measurement of two- and four-electrode method (WennerAlpha type), it was investigated how much the electrode impedance contributes to the total measured impedance (electrode characterization is performed by Dipl.-ing. Christian Kurz from workgroup "Biohybrid System" of Fraunhofer IBMT). It was not possible to record bio-electric signals from the developing chicken embryo cultured in modified open system with the help of implantated flexible electrode array due to short of time.

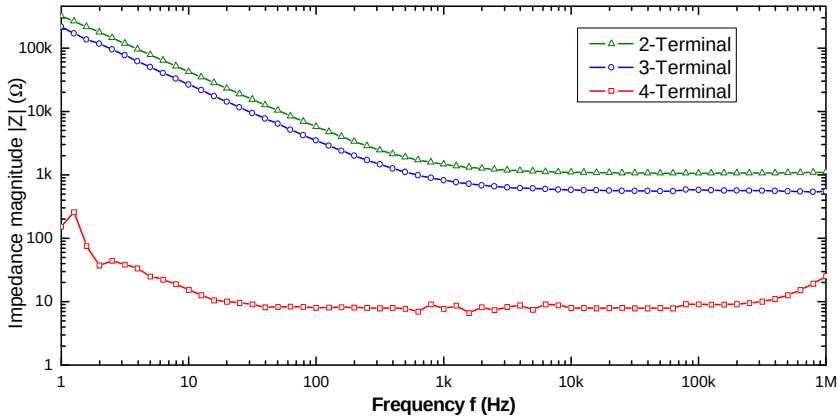

Figure 5.14: Impedance spectra of PBS electrolyte at room temperature measured by two, three, and four-electrode method with fabricated electrode array

5.5.3 Study of the stability of the open system with implantation of a platinum-polyimide micro-electrode

Even though the biocompatibility of such platinum-polyimide microelectrode is proven and routine animal experiments are performed using such electrodes, but the scenario could be different in the developing embryo. Since the hatching of a viable chick from the open avian culture system was set as the gold standard to asses the functionality of the system, it was necessary to asses the effect of the implanted electrode on hatchability before the electrical signal measurement.

The flexible Pt-polyimide electrodes implantation into the surrogate/recipient egg was performed a day before the embryo transfer in the intact donor egg. A point close to opening of the recipient egg was marked as the location for electrode implantation. Egg shell from the selected region was ground off from ≈2 mm diameter circle leaving the egg membrane intact. The egg was than rinsed with distilled water to remove egg shell powder and wiped with sterile paper towel. The electrode tip was held with a pair of forceps and implanted through the shell membrane. The hole was than sealed with

silicone. Distilled water was sprayed on the egg and the egg was than returned to the refrigerator at 14 °C temperature. Silicone releases Acetic acid during the process of polymerizations which will certainly change the P^H of the culture environment and may have detrimental effect on the embryonic development. That is why the electrode placement was performed in an intact egg with its contents inside and acetic acid produced during polymerization of the silicone was diluted in the egg white which was discarded later during the shell transfer. The egg was thoroughly rinsed with distilled water again on the next day before the embryo transfer procedure.

On the following day the surrogate shell was prepared as described in chapter 4.5.1. Three days preincubated bantam chicken embryos were transferred into the surrogate shell containing the electrode. The opening was closed with a lid / cling film and the whole construct was incubated at 37.5 °C temperature, 60% RH and 30° side to side rocking.

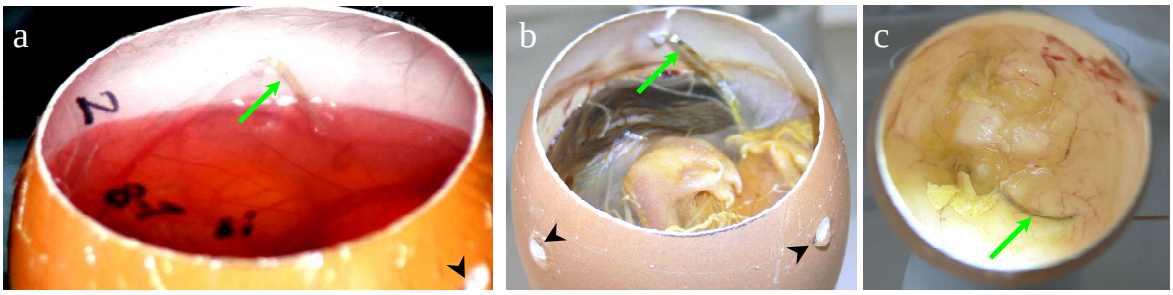

Figure 5.15: Platinum-polyimide electrode implanted in the open system.

Bantam chicken embryo in surrogate shell with implanted Pt-polyimide electrode at (a) ID10 and (b) ID 20. Note the close proximity of the electrode to the embryonic body, which is essential for high SNR. (c) Surrogate shell after removal of the embryo at ID 20 shows the electrode position underneath the CAM (green arrow indicates electrode, black arrowhead indicate the holes made on surrogate shell for insertion of electrode sealed with silicone).

Figure 5.16 shows the result of the culture of bantam chicken embryos in open system containing Pt-polyimide electrode. 75% embryos survived until hatching. In comparison to the control culture in chapter 4.5.1 (≈67%) the results show that there is no significant influence of the electrodes on the hatching of the chicks. Figure 5.15 shows the image of the embryo culture where the electrode is integrated into the CAM and closely associated to the developing embryo. Such close contact is essential to measure very low amplitude electrical signals like ECG in the early days of embryogenesis. Because of the above mentioned advantages of flexible electrode, it will ease the measurement of electrical signal throughout the whole developmental period

especially at the end of incubation when the pulmonary ventilation is activated. It should bring less motion artifacts and noises as mentioned by different investigators where they placed small electrode between the egg membrane and CAM since the electrode was flexible and can move along with the embryo. This method also avoids injury to the CAM because the electrode was implantated before the formation; CAM incorporates the electrode within during formation improving the SNR.

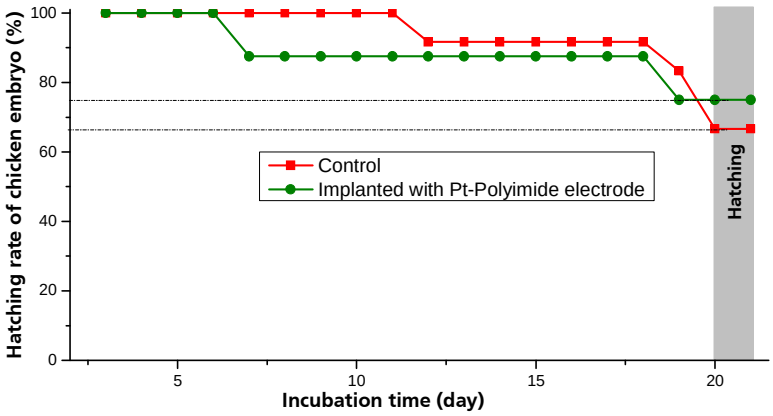

Figure 5.16: Viability of bantam chicken embryo cultured in open system implanted with Pt-Polyimide electrode.

75% embryos survived until day 21, which correlates with the data with the control experiment in chapter 4.5.1 (≈67% viability at day 21).

5.6 Discussion

The method of explantation culture in surrogate shell has long been used for the production of transgenic birds by the biologists. However, this was not used to study the process of targeted cell differentiation that happens during embryogenesis. The study of embryogenesis is easier in avian embryos over mammals. The avian embryos are complete in terms of the nutritional and other requirements. Mammals are completely dependant on their mother. It is necessary to have insights into the developing embryo to understand the cellular microenvironment essential for linage specific differentiation of stem cells, which is not yet possible *in vitro*. It was necessary to modify the existing open culture system for optical imaging methods. In addition, it was also necessary to construct special systems for *in vivo* application. With the both, it was possible to observe the whole period of embryonic development in a very high resolution. Such experiments involving mammals are impossible.

Since the imaging of the embryonic development was performed without contrast labelling, it was not possible to distinguish between different cell groups. Enhancement of contrast by some means is necessary for studying the processes of cell differentiation and migration. This obstacle can be removed by the injection of contrast labelled cells into the embryo and following their fate with the long distance fluorescence micro-imaging system. Further experiments are necessary in this field. Different cell labelling techniques combined with modified open system may reveal many secrets regarding stem cell.

Several studies have shown that mammalian cells and tissues transplanted to avian embryos can respond to local cues and develop into tissues appropriate to their location in the host [93, 106, 385]. Hematopoietic stem cells (HSCs) from adult human bone marrow Implanted into lesions of the developing spinal cord in the chicken embryo differentiated into full-fledged neurons but never express a chicken-specific antigen. This suggests that the microenvironment in the regenerating spinal cord of the chicken embryo stimulates substantial proportions of adult human HSCs to differentiate into neurons [304]. Such targeted differentiation of stem cells *in vitro* is not yet possible until today. Therefore, the investigation regarding the cellular microenvironment *in ovo* could reveal secrets behind the differentiation of stem cell, which is not possible, *in vitro*.

Although the especially designed flexible electrode array was constructed and characterised, it was not possible to record bio-electric signals due to the shortage of time. Measurement of bioelectric signals from the developing embryo could provide with valuable information. Until now, most of the experiments regarding bioelectric signal acquisition have been concerned with the middle and the later part of the development of chicken embryo. Because of feeble electrical signal from the primordial heart in the early stage of incubation, it is difficult to measure the ECG in the early stage of development. However, the flexible Polyimide-platinum electrode array was designed keeping this special interest in mind. Preliminary experiments with implanted electrode in open culture system showed no significant effect of the electrode array on hatching rate of the chick (Figure 5.16). The flexibility of the electrode array will ensure the close apposition of the electrode to the embryo without injury and improve SNR.

This electrode can be applied to study the evoked potentials (visual, auditory and others) of the developing embryo. Characterisation of embryonic development with impedance spectroscopy is of especial interest in this field. The avian egg consists of lipid

nucleus (egg yolk) surrounded by albumen rich in water. Both the egg yolk and the egg white are consumed by the developing embryo, which will change the electrical impedance of egg contents. Characterization of a developing chicken embryo with impedance spectroscopy will provide valuable information in this field. It is necessary to further investigate in this field to exploit the advantage of impedance spectroscopy.

5.7 Outlook

Experimental investigations in this chapter were mainly concerned with the preparation for the future experiments that will explore the world of cell differentiation *in vivo*. This involved the modification of the open culture system applicable for optical imaging, construction of especially designed fluorescence micro imaging and micromanipulation system for *in vivo* application, construction of flexible electrode array *in ovo* application. It was possible to image the whole period of embryonic development of a chicken embryo from the beginning of the incubation until hatching using the developed system. Even though it was not possible to use the especially designed flexible electrode array for acquisition of bioelectric signal and impedance measurement due to shortage of time, but the electrode is characterised and implantation into the modified open system showed that there is no significant effect on the hatchability. Further experiments are needed in this field in future. The whole system can be employed to monitor the process of embryonic development, to reveal the secret behind organised cell migration and differentiation that makes a living organism from a single cell.

6 Gradual technical modifications of the open system towards liquid │liquid interface culture

6.1 Summary

Objective: The experimental investigations in this chapter were aimed to investigate the functionality of the open system as a liquid │liquid interface culture system and to identify different influential parameters, especially to study the dynamic nature of the eggshell as a culture system, which adapt the boundary conditions synchronously and permanently in accordance with the changing need of the developing embryo. Based on results of the experiments in this chapter, some suggestions are made at the end of the work that will help in future to design newer ways to realise a liquid │liquid interface culture system for culturing mammalian cells at the interface of two immiscible liquids. The final system may not be similar in geometry to the avian egg but will use the same principle for culturing cells.

Methods: This chapter includes the studies of the eggshell and shell membrane with scanning electron microscopy to reveal the ultra structure, study of the biocompatibility of unknown materials, replacement of part of the surrogate shell with biocompatible, optically transparent materials gradually to study the influence of different materials on the growth of CAM in the open system. Based on the results of these experiments, different model systems were constructed that applied fluidics for culture medium exchange and channels for monitoring of gaseous environment and exchange.

Results: The shell membrane is porous and composed of very fine fibrous structures that separate the egg contents (especially CAM) from being in direct contact with calcareous egg shell. It was not possible to replace the eggshell with the available materials. At the end of this chapter, different models are constructed for the demonstration of the potential of chicken egg.

Conclusions: The results of the experiments demonstrate the dynamic nature of the eggshell. It was not possible to replace the eggshell with available materials having static propertyies. It will, therefore be necessary in future to adjust the culture environment in accordance with the changing need of the growing embryo with feedback control using

different biosensors. It is also necessary to search for materials with adjustable properties for the future experiments.

6.2 Materials required for gradual technical modifications of the open system towards liquid | liquid interface culture

Equipments	Manufacturer
Fluidic panel	Evotec technologies, Germany
LKB 2249 gradient pump	Bromma, Sweden
Top-Profi 240 egg incubator	Hemel Brutgeräte, Germany
Chemicals	**Manufacturer**
Amphotericin B (250 µg/ml)	Fisher Scientific, USA
Bacillol AF disinfections solution	Bode Chemie, Hamburg
ECM	Merck, Darmstadt
Fibronectin	Merck, Darmstadt
Gelatine	Merck, Darmstadt
Hypochloride solution (200-500 ppm)	VWR International GmbH
PBS	Invitrogen, Karlsruhe
Poly-L-Lysin	Merck, Darmstadt
P/S Penicillin/ Streptomycin	Invitrogen Corporation, USA
Silicone adhesive	NuSil Technology, USA
Biological agents	**Manufacturer/supplier**
Fertilised bantam chicken eggs	Anita Nefzger, Leutershausen
Fertilised White Leghorn chicken eggs	LSL Lohmann Tierzucht GmbH. Cuxhaven
Fluoresceindiacetate (FDA)	Invitrogen, Karlsruhe
L929 Mouse fibroblast cells	DSMZ, Braunschweig
Unfertilised broiler eggs	Glückliche Eier, Saarbrücken
Accessories	**Manufacturer/supplier**
Bioglass (FluoroDish)	World precision instruments, USA
Cell culture petridish	Greiner Bio-One, Frickenhausen
Egg shapped plastic container	GALERIA Kaufhof, Saarbrücken
ITO glass	Präzisions Glas & Optik, Germany
Polyamide	Fraunhofer IBMT
Plexiglas	Alois Schmitt GmbH, St. Ingbert
Thermonox® Plastic Coverslips	Nunc, Langenselbold
Computer Software	**Developer**
LabVIEW	National Instruments, USA

6.3 Background

The eggshell is a dynamic culture system, which is changes the property permanently during the whole period of embryonic development and adapting according to the need of the developing embryo. At the beginning of incubation, the internal environment *in ovo* is rich in CO_2, which is essential for the avian embryo at an early stage. This is the reason behind the pre-incubation of the embryo inside egg before transfer into surrogate shell; otherwise, it has to be cultured in CO_2 rich environment. As the embryo grows, O_2 becomes essential. The eggshell gradually adapts, increasing of the gas exchange. This is achieved by gradual thinning of the shell by mobilisation of Ca^{2+} CAM. This also increases the rate of evaporation of H_2O across the eggshell which is necessary for the growth of the embryo. The Chicken egg is a dynamic system which regulates water loss about 380-490 mg/day [15]. About 10 to 11% of the water is lost through egg shell and shell membrane in domestic fowl eggs during the incubation period of 21 days [354]. This water loss is not constant; it increases as the eggshell thins.

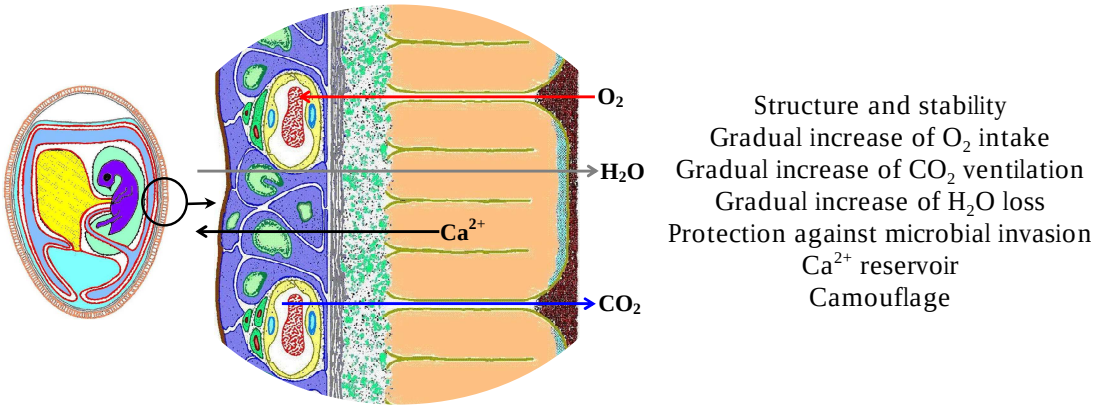

Figure 6.1: Schematic representation of egg shell functions.

6.4 Influences of different physical conditions of the surrogate shell on the viability of the chicken embryos

The aim of the experiments was to find out the influence of different physical conditions of surrogate shell like drought and removal of shell membrane on developing chicken embryo. Scanning electron microscopy of eggshell and shell membrane was performed to find ultra structural changes of the eggshell and shell membrane in different physical state.

6.4.1 Culturing chicken embryos in surrogate shells without the shell membrane

Aim of this experiment was to find the effect of calcareous eggshell without shell membrane on the viability of chicken embryos. Eggshell is the main source of Ca^{2+} for the developing embryo. However, the calcareous eggshell is totally isolated from the CAM responsible for calcium absorption from the shell.

For this experiment, the surrogate shells were prepared as described in Chapter 4. The inner and the outer shell membranes were stripped off from the surrogate shell leaving the calcareous eggshell bare. The eggshell without the shell membranes was rinsed with distilled water. Transfer of the 36 hrs old embryo and incubation was preformed as described in Chapter 4.

6.4.2 Effect of egg shell drought on the viability of chicken embryos

The aim of this experiment was to study the effect of drought of surrogate shell on the viability of the avian embryo. As routine procedure for explantation culture (Chapter 4), the donor egg was emptied of the egg contents to prepare the surrogate shell, which is washed with distilled water to prevent drought before transfer of the preincubated embryo.

For this experiment, the surrogate shells were prepared as described Chapter 4. The inside and outside of the shell was thoroughly rinsed with distilled water to remove egg white and chalazae. The clean shells were then placed inside a laminar flow cabinet on a custom-made holder with the opening placed upwards and kept there for 24 hrs to dry. On the following day, the dried shells were thoroughly washed and wetted with

distilled water, filled with thin egg white taking care not to spill and kept for half an hour. Egg white was than discarded. Transfer of 3-day-old chicken embryo to the dried surrogate shell and incubation was carried out as described in Chapter 4.

Figure 6.2 shows the effect of different physical conditions- drought and removal of shell membrane from surrogate shell on viability of the chicken embryos. It is important to note that no embryos survived beyond ID 5 when cultured in bare surrogate shell without shell membrane. Figure 6.3 shows the growth of the CAM in explantation culture in complete transparent system.

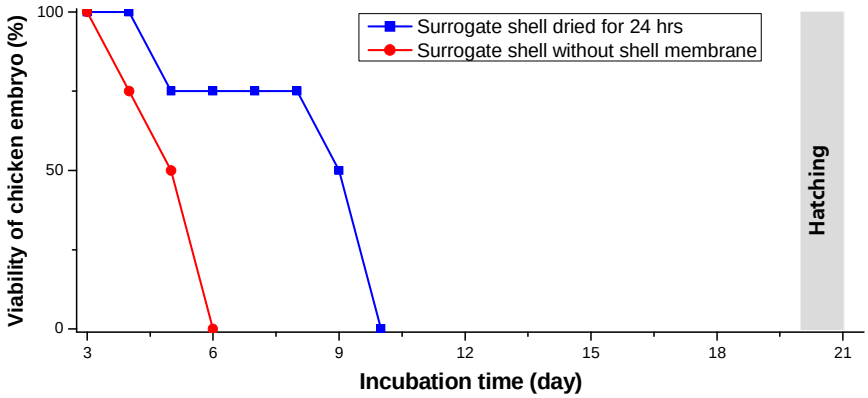

Figure 6.2: Effect of different physical conditions- drought and removal of shell membrane from surrogate shell on viability of the chicken embryos.

When cultured in bare surrogate shell without shell membrane, no embryo survived beyond ID 5. In case of drought of surrogate shell, maximum embryo survival was ID 9.

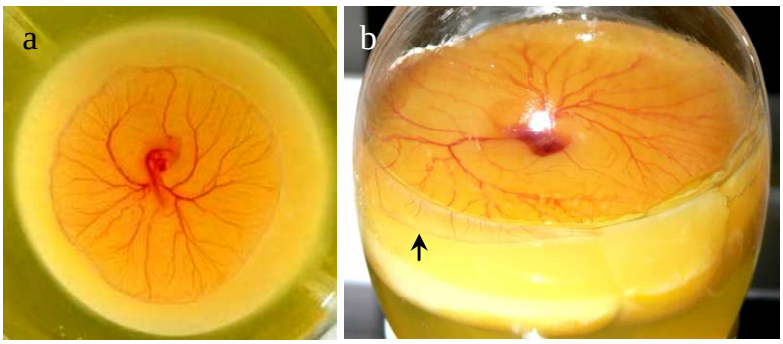

Figure 6.3: Growth of the CAM in open culture consisting of a completely transparent system

Chicken CAM at (a) ID 4 and (b) ID 5. Notice by ID 5 the CAM grows and touch the side wall of the explantation culture system (arrow in image b marks the CAM boundary).

It is clearly visible that by ID 5, the CAM touches the calcareous wall. Eggshell is known to triggers inflammation in the CAM [163]. Since in surrogate shell culture, the shell membrane remains intact, and the CAM doesn't come into direct contact with the calcareous shell. In this experiment the shell membrane was removed and by day 5 the

CAM comes in direct physical contact with the calcareous shell. This could be the reason that all the embryos were dead by day 6 of incubation. However, when cultured in dried surrogate shell, t embryo survival was longer, ID 9. There was a sharp decrease of viability on day 8 of incubation and on the next day, all embryos died. To investigate the fact further, scanning electron microscopy of the dried eggshell and the egg membrane was performed.

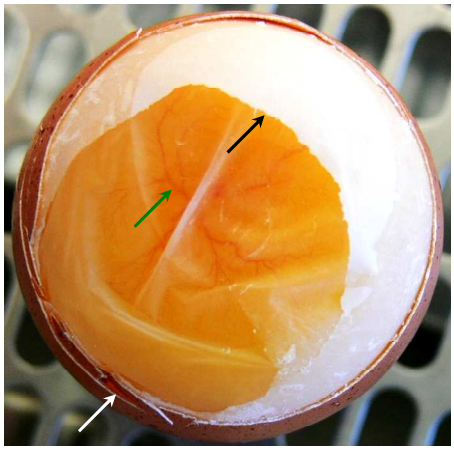

Figure 6.4: 36 hrs old chicken embryo after removal of eggshell and outer shell membrane from the blunt pole of a chicken egg.

Inner shell membrane is kept intact for demonstration. Note that the egg contents are completely isolated from the calcareous shell (white arrow) by inner and outer shell membrane (black arrow). Green arrow indicates the developing embryo inside inner shell membrane.

6.4.2.1 Scanning Electron Microscopy (SEM) of chicken eggshell and shell membrane

For SEM of eggshell and shell membrane, samples were taken from freshly laid eggs of Rhode Island Red chicken. Samples were washed thoroughly in distilled water to remove traces of egg white. Eggshell with shell membrane was dried in air before sample preparation.

The samples were fixed with a cacodylat-based fixation buffer. Afterwards they were incubated with a cacodylat solution, followed by osmium buffer and tannin acid-incubation. After dehydration with ethanol the samples were analyzed by “LEO 435 vp” scanning electron microscope at 15.0 KeV (the preparation of the samples for scanning electron microscopy was done by Dipl.-Leb.Chem. Yvonne Kohl from the workgroup “Biohybride system” of Fraunhofer IBMT and the imaging was done in Zweibrücken, Germany at the “department of Informatics and Mikrosystemtechnik” of FH Kaiserslautern by Dipl. Ing. Rainer Lilischkis).

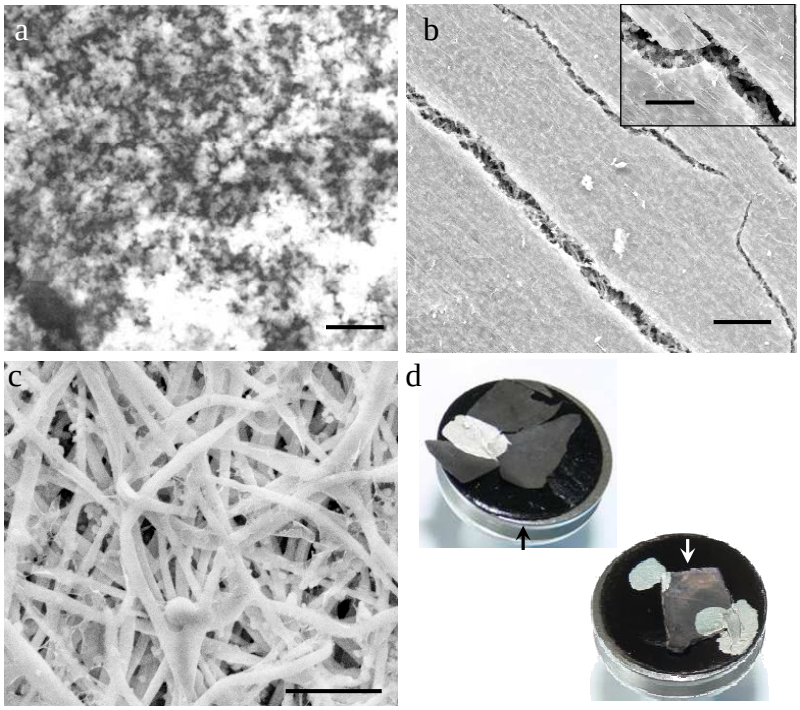

Figure 6.5: Scanning electron microscopic image of eggshell and shell membrane of Rhode Island Red chicken egg.

(a) Egg shell from outside, (b) egg shell from inside along with shell membrane; (c) acellular inner shell membrane, (d) prepared sample for scanning electron microscopy (black arrow = egg shell membrane, white arrow = inner side of the egg shell along with membrane). Note in image (b) furrows appeared in the shell membrane due to drought during preparation which can be better observed in the inset. In image (c) the fibrous structure of the shell membrane is visible. Scale bar (a) = 10µm; (b) = 150 µm; (c) = 50 µm; inset in (b) = 50 µm.

Figure 6.5 shows the SEM image of the dried eggshell and shell membrane. Notice the fibrous and porous structure of the shell membrane. There are creases in the shell membrane located at inner side of the eggshell in image (b) which was probably due to the shrinkage of the egg membrane due to dehydration. These furrows expose the calcareous eggshell to CAM. This explains the result of the experiment showed in Figure 6.2. Since the CAM was minimally exposed to the calcareous eggshell through the furrows on the dried shell membrane, maximum survival of chicken embryos were longer (day 9) than the previous experiment where the embryo was cultured in surrogate shell without shell membrane (Figure 6.2).

6.5 Windowing of the surrogate shell with transparent biocompatible materials

The idea of this windowing experiment was to find out the property of different materials, to see their effect on the growth of CAM and their effect on hatchability. This can help to identify the appropriate material for construction of complete artificial system. Since the opaque calcareous eggshell is not suitable for optical imaging, it should be replaced with transparent biocompatible materials suitable for *in vivo* application. In addition, this can be used for illumination for optical imaging or observation window. In this experiment, part of the eggshell was removed and replaced by different optically transparent, biocompatible materials. For unknown materials, the sample was coated with different extracellular matrix (ECM) protein and amino acids and biocompatibility was examined with fibroblast proliferation assay. There is no scientific data available in this regard and it was very important to understand different properties of the egg shell to allow reconstruction of the whole system artificially.

6.5.1 Tests for biocompatibility: material coating and fibroblast proliferation tests

Different materials were used for windowing experiments, these were traditional cell culture Petri dishes, Polyimide membrane, plastic cover slips, Bioglass (taken from FluoroDish™ from World precision instruments), CEC020S Indium-Tin-Oxide (ITO) coated glass (From Präzisions Glas & Optik GmbH, Germany), Plexiglas. Most of the materials are routinely used for cell culture. ITO coated glass was subjected to cellular proliferation test (Fibroblast proliferation assay) (the biocompability experiments were performed by M.Sc. Ina Meiser from "Biophysics & Cryotechnology department" of Fraunhofer IBMT).

ITO coated glass was coated with different extracellular matrix (ECM) protein and amino acids like (a) 0.01% Fibronectin in PBS, (b) 1:20 ECM in culture medium (c) 0.1% Gelatin in PBS (d) 0.1% Poly-L-Lysin in PBS assessed for cellular proliferation. As a cell system, L929 Mouse fibroblasts are used. The cellular proliferation process was monitored over 24hrs in Nikon Biostation CT.

Figure 6.6 shows the results of fibroblast proliferation assay of ITO glass. From the result, it is evident that there is no significant difference between treated and non-

treated surface (a in Figure 6.6 is the control - without coating). It can be concluded that ITO coated glass has no toxic effects to cells and it is biocompatible.

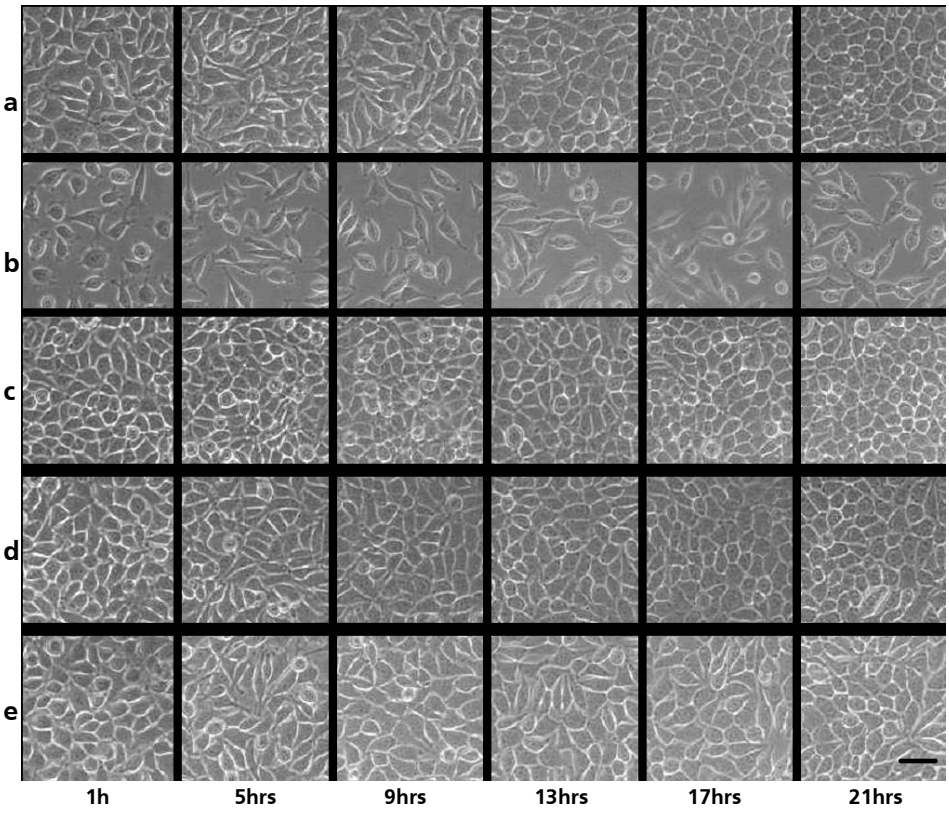

Figure 6.6: Results of material coating & cell proliferation test for CEC020S ITO coated glass.

(a) control (without coating); (b) 0,01 % Fibronectin in PBs, 30min Incubation time at 37 °C; (c) 1:20 ECM 30min Incubation time at 37 °C; (d) 0,1% Gelatine 1h Incubation time at 37 °C; (e) 0,1% Poly-L-Lysin in PBS, 30min Incubation time at 37 °C. Results show no significant difference between coated and non-coated surface of ITO coated glass. Scale bar = 50 µm.

6.5.2 The windowing experiment

In windowing experiment, part of the surrogate shell was replaced with different biocompatible and transperant materials and used for bantam chicken embryo culture. The eggshell was removed from 3.14 cm^2 (Ø 20 mm) area at different locations of the surrogate shell and the window material was glued with medical grade silicone. Along with the biocompatibility of the window materials, the window area is also crucial for the growth of the embryo. Because in open system, the CAM is in contact with natural shell membrane of the surrogate shell, and no part of CAM was in contact with artificial materials. The upper surface of CAM was not in contact with the shell membrane and not taking part in the normal CAM functions necessary for embryonic development, especially calcium transport and gas exchange (Figure 4.15). The opening diameter of the surrogate shell was ≈45 mm (Ø) which is nearly 15.89 cm^2, that corresponds to

approximately 18% of the total CAM surface of about 87 cm^2 on 10^{th} day of incubation [32]. It was also considered that additional removal of bioactive surface from the CAM might be detrimental to the growth and development of the embryo.

6.5.3 Preparation of the surrogate shell for windowing experiment

Recipient eggs were chosen for preparation of surrogate shell as described earlier (Chapter 4). The recipient egg shell was wetted with distilled water. At different region of the egg, the shell was ground-off for a 20 mm circular area keeping an egg shell island in the middle surrounded by the intact shell membrane. The egg was thoroughly rinsed with distilled water to remove traces of egg shell powder. The ground region of the recipient shell was thoroughly checked for any shell remains; if there were any, cleaned again so that no shell parts remained on the shell membrane. The egg was then placed in horizontal position with the window location upwards. The shell island in the middle surrounded by shell membrane was carefully cut with a scalpel and removed with a pair of forceps leaving the egg membrane intact.

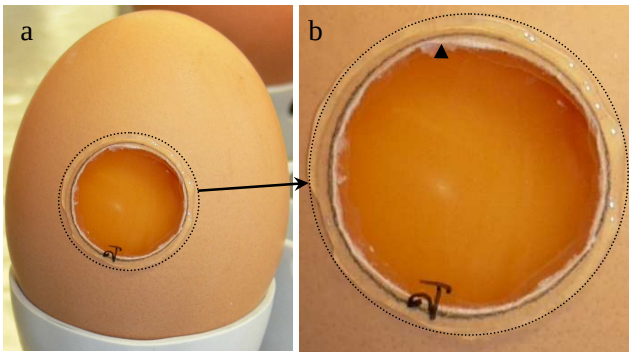

Figure 6.7: Surrogate shell preparation for windowing experiment.

(a) The prepared shell with its contents; (b) zoomed view at the window shows the shell membrane is hanging beyond the shell (arrow head) to prevent CAM contact with the calcareous shell.

Medical grade silicone was applied at the rim of the window prepared in the recipient egg to cover the bare end of the egg shell margin that no part of the ground shell margin remains uncovered. The window material was applied on the window opening and pressed firmly in such a way that the window became airtight. The egg was kept in horizontal position with the window facing upwards until the glue dries. Then the egg was returned to the refrigerator at 14 °C with long axis vertical (Figure 6.7) and stored there for next 24 hrs. On the next day, the surrogate shell was prepared,

36 hrs old chicken embryos were transferred to the windowed surrogate shell and incubated as described earlier (Chapter 4).

6.5.4 Results of the windowing experiment

Figure 6.8 and Table 6-1 shows the summerised results of chicken embryo cultured in surrogate shell open system windowed with different biocompatible materials. Although most of the materials are biocompatible and used routinely in the laboratory for cell culture, the experiment results were not satisfactory. The window area covered only 4% surface ares of CAM at ID 10. This indicates that the material properties should identical to the shell membrane for constructing a completely artificial egg.

Table 6-1: Summarised results of windowing experiment with different biocompatible materials

Material	Total sample	Max. survival	Window location	Comment
Polystyrene coverslips	10	Day 20	Mid-equatorial region	CAM released or blood vessel disappeared from the window before death, regular pattern of blood vessel growth
Bioglass	10	Day 17	Mid-equatorial region	CAM didn't grow well on bioglass, regular pattern but incomplete growth of CAM blood
Polyamide	10	Day 14	Mid-equatorial region	blood vessel disappeared from the window before death, irregular pattern of blood vessel growth
Cell culture petridish	10	Day 20	Pointed end	blood vessel disappeared from the window before death
Cling film	10	16	Pointed end	Viability decreased sharply at day 6
Plexiglas	10	15	Pointed end	Viability decreased sharply at day 6

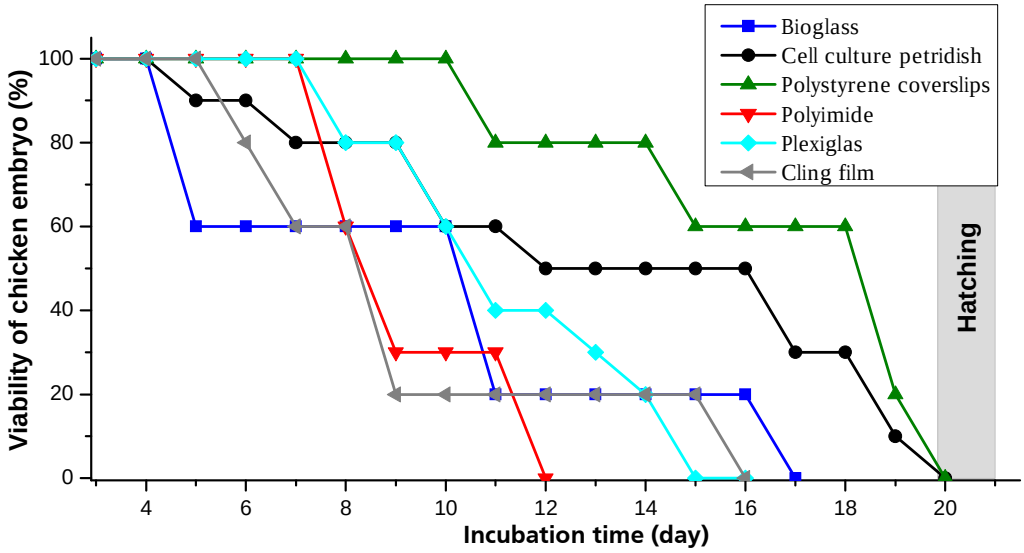

Figure 6.8: Results of the culture of chicken embryos in surrogate shell windowed with different biocompatible materials.

Among all investigated materials, bioglass and polystyrene cover slip had better results if the maximum viability of the embryo is concerned. Embryos grown in surrogate shell windowed with polystyrene cover slip had maximum viability at day 18 of incubation (≈60%). Besides, CAM growth on the polystyrene cover slip window was also normal and CAM blood vessels gradually grew from above to below and eventually covered the whole widow surface (Figure 6.10). On the other hand Polyimide membrane (even though used for manufacturing of implantable electrodes) and cling film (used to cover the surrogate shell opening) had worst outcome among all.

Bioglass

Figure 6.9 shows the outcome of culturing chicken embryos in surrogate shell windowed with bioglass (taken from FluoroDishTM from World precision instruments).

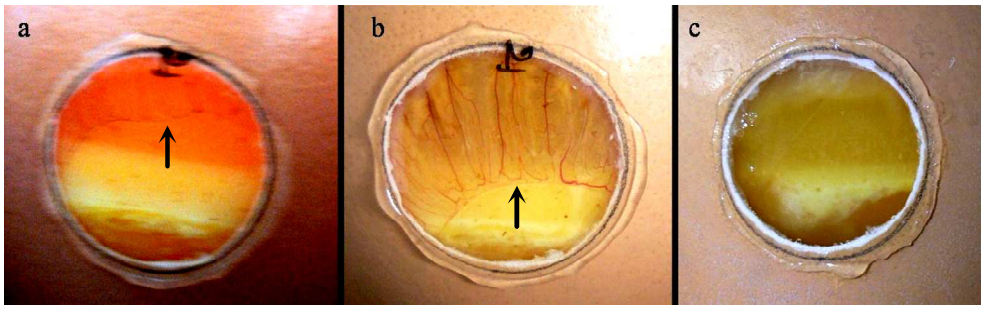

Figure 6.9: Growth of chicken embryo in windowed surrogate shell with bioglass taken from FluoroDish™ (Ø20mm).

(a) ID 7, (b) ID 11, (c) ID 12. The maximum viability embryo in this system was ID 11; embryo died on the next day which is evident by disappearance of CAM blood vessels. Note the growth of CAM was not complete (which is evident by the visible lower limit of the CAM [the lower limit of CAM is marked by black arrow in the image (a) and (b)]. But CAM blood vessels grew in normal parallel pattern.

The CAM grew on bioglass gradually from above to down. The lower margin of CAM is marked by black arrow (a-b in Figure 6.9). However, the CAM growth was not complete. Eventually the CAM blood vessels disappeared from the window at ID 12 indicating the death of the embryo.

Polystyrene

Figure 6.10 shows the growth of the CAM of developing chicken embryo through the polystyrene window in surrogate shell. CAM gradually grew from above to below and eventually covered the whole window by ID 16. The pattern of the CAM blood vessels was normal. At ID 19, CAM blood vessels disappeared from the window indicating the death of the embryo.

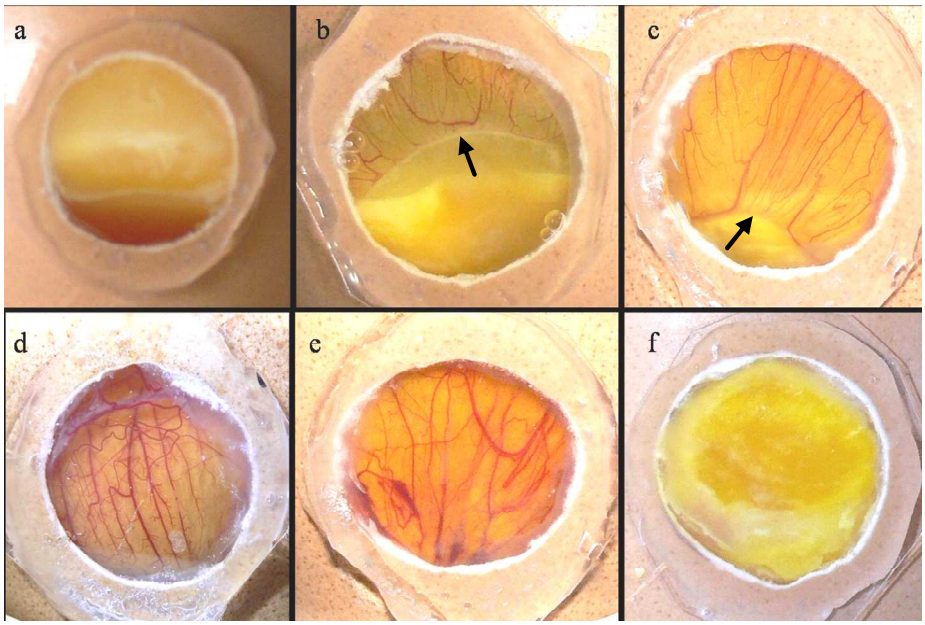

Figure 6.10: Growth of chicken embryo in windowed surrogate shell with polystyrene cover slip (Ø20mm).

(a) ID 6, (b)11th day, (c) ID 14; (d) ID 16; (e) ID 18; (f) ID 20. Note gradual growth of CAM from up to downwards and eventually covered the entire window surface with normal pattern of CAM blood vessels. Maximum survival was ID 19 (e), afterwards blood vessels disappeared and eventually the embryo died. In some of the cases, the CAM was released from the Polystyrene window and eventually the embryo died (Figure 6.11).

In some cases, the CAM was separated from the window after growing (Figure 6.11). This separation of CAM from the window in surrogate shell led to the death of the embryo. This indicates that the window material was not favorable for CAM attachment.

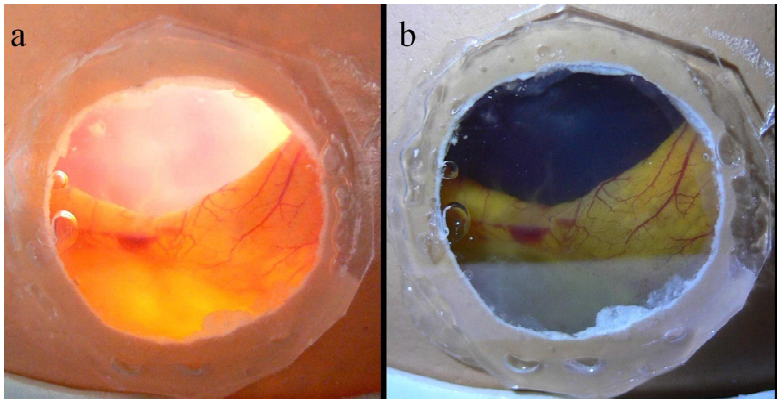

Figure 6.11: Growth of chicken embryo in windowed surrogate shell (Ø = 20mm) with polystyrene cover slip.

Note the CAM is separated from the Polystyrene cover slip at 14th day of incubation. This indicates that CAM couldn't attach firmly to the window though the polystyrene cover slips are routinely used for cell culture in the laboratory. (a) Trans-illumination, (b) reflection imaging.

Polyimide

In Figure 6.12 the growth of CAM on polyimide membrane is shown. The pattern of the CAM blood vessels on polyimide membrane was not regular and the CAM didn't grow completely. At ID 12 the CAM blood vessels disappeared indicating the death of the embryo.

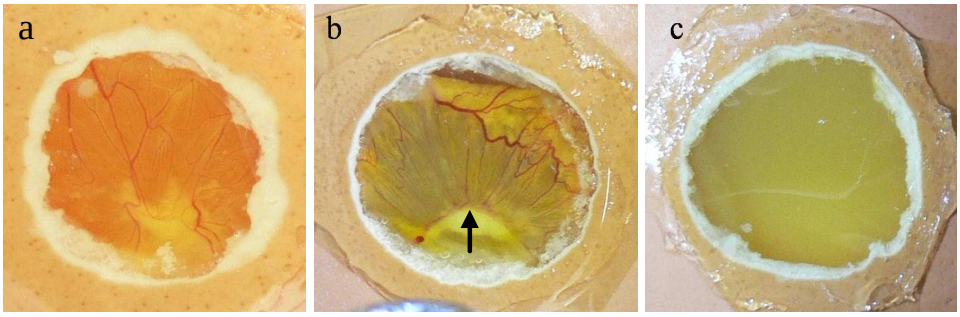

Figure 6.12: Growth of chicken embryo in windowed surrogate shell with polyimide membrane (Ø10mm).

(a) ID 6, (b) ID 11 and (c) ID 12. Note the irregular pattern of CAM blood vessel growth from the beginning and the CAM did not grow completely on Polyimide membrane. Maximum viability was day 11 of incubation.

Cling film (polyvinyl chloride)

Figure 6.13 shows the preparation of windowed surrogate shell with cling film and Plexiglas at the pointed end and culture of chicken embryo in the windowed system. Even though the maximum viability of the chicken embryos in this system ID 16 for cling

film and ID 15 for Plexiglas, there were a sharp decrease in viability of the embryos at ID 6.

Figure 6.13: Growth of chicken embryo in windowed surrogate shell with cling film and Plexiglas (Ø30 mm).

Image (a-c): Cling film window and (d) Plexiglas-window at the narrow end of the surrogate shell. (c) Chicken embryo in surrogate shell with cling film window at ID 8. Maximum embryo survival in such windowed system was ID 16 for cling film and ID 15 for Plexiglas. Viability of the embryo decreased from ID 6 and ID 8 sharply for cling film and Plexiglas windowed surrogate shell accordingly.

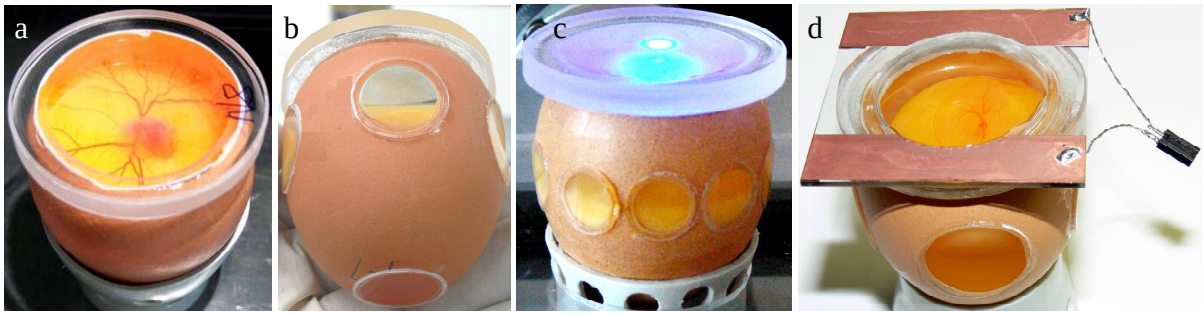

Figure 6.14: Examples of different modifications of open system of avian culture for future considerations

(a) 10 mm window at the pointed end with Plexiglas-silicone lid (not visible in the image); (b) multiple 20 mm window on the side and bottom with Plexiglas-silicone lid; (c) 30 mm windows on the side with Plexiglas-silicone lid with ITO heating glass; (d) multiple 10mm windows circumferentially with Plexiglas-silicone lid. Image (a) contains 96 hours old chicken embryo, others contain 36 hours old chicken embryos.

Figure 6.14 shows some different variations of windowing experiment that could help to design further investigations in future to asses the material property. Even though the complete experiments were not conducted in such systems, but these can be used as examples for future experiments with different window materials that can help to find the appropriate materials for construction of completely artificial systems in the future.

6.6 Installation of fluidics and channels into the open system for exchange of culture medium and gas

To fulfil the final goal of this thesis, natural avian eggs are technically modified and finally reconstructed for culturing cells at the liquid | liquid interface. It is necessary to exchange the culture medium, substrate and gas which requires fluidic channels installed in the system. Figure 6.15 shows the model where the fluidic inlet and outlet channels are installed into the surrogate shell that can be used for exchange of culture medium. Additional channels can be installed for exchange of substrate if necessary. It was not possible to conduct the complete experimental investigations into the feasibility of such installation. Nevertheless, this can be an outline for future investigations.

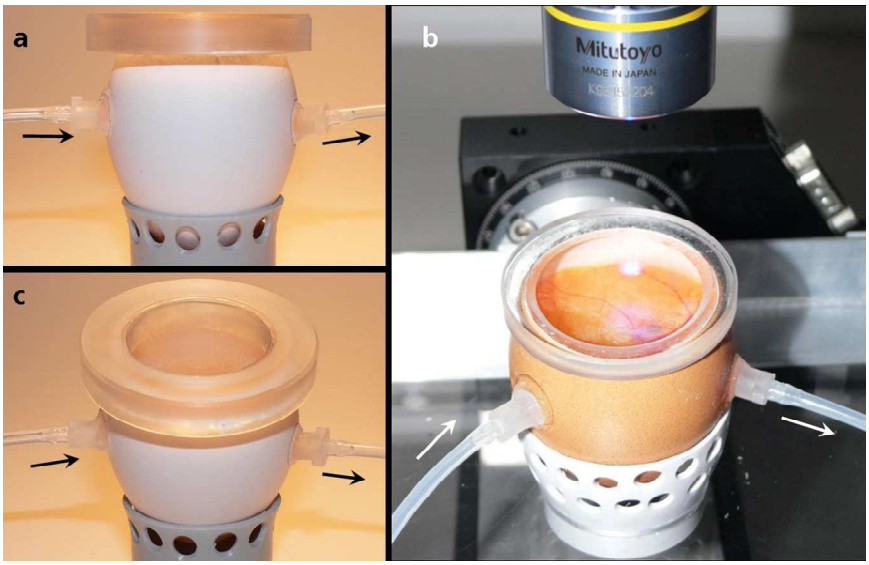

Figure 6.15: Application fluidic channels in surrogate shell for addition and removal of culture medium.

Gradual adaptation of the open system of avian culture for in vitro culture of cells: application fluidic channels for addition and removal of culture medium. (a) and (c): modified open system consisting of White Leghorn egg shell, PMMA double glass cover with inlet and outlet channels; (b) bantam chicken embryo at ID 5 inside Rhode Island Red egg shell modified system with fluidics and PMMA cover.

However, it is necessary to asses the feasibility of such system in terms of the viability of chicken embryo. As described earlier, the hatching of a viable chick was regarded as the standard for assesing the functionality of the system. Even though it is not necessary to have any extra means for the avian embryo, since it is a complete system, self equipped with everything that is necessary for a developing embryo. However, culturing cells at the liquid | liquid interface will certainly require for exchange of culture medium and substrate. In such cases, exchange of culture medium and substrate through existing fluidics will be more convenient.

6.7 Completely artificial and transparent eggs

It is necessary to understand all the boundary conditions to enhance reproducibility, when constructing a completely artificial system for culturing cells at the liquid | liquid interface. It is advantageous to have completely artificial materials instead of natural eggshell. It is advantageous to have a completely artificial and transparent egg, easy to observe with optical imaging methods. Stepwise technical modification is necessary for complete replacement of the avian egg; change of too many parameters at once will lead to inappropriate trouble shooting.

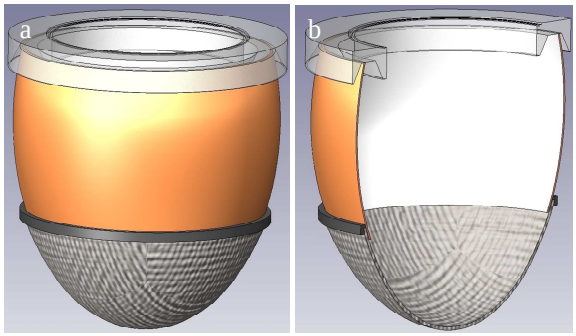

Figure 6.16: Computer simulation of a hybrid (semi artificial) egg.
Such hybrid /semi artificial system consist of a part of the natural eggshell and suitable artificial materials.

Figure 6.16 shows a hybrid egg where the lower part of the eggshell is replaced with artificial material. It is necessary to investigate the properties of material suitable for *in ovo* application. The windowing experiment in the section 6.5 of the current chapter may provide with some clue in this regard. The idea behind stepwise modification of the complete natural functioning system is to identify the problems. If too many parameters are changed at a time, it will be difficult to identify the reason behind the non-functionality of the system.

Figure 6.17 shows some examples of completly artificial eggs. Although until now, it was not possible to culture avian embryos to hatching or culture cells at liquid | liquid interface in such system, further investigations may overcome these obstacles. Image (f) in Figure 6.17 shows the example of such artificial eggs with installed fluidics and channels for culture medium and gaseous exchange. Please note that these models are constructed with the outline of the previous experimental investigations conducted earlier in this thesis so that it will help to extend the imagination in future. Figure 6.18

shows a complete outline of the experimental setup for in vitro culture of cells at the liquid | liquid interface.

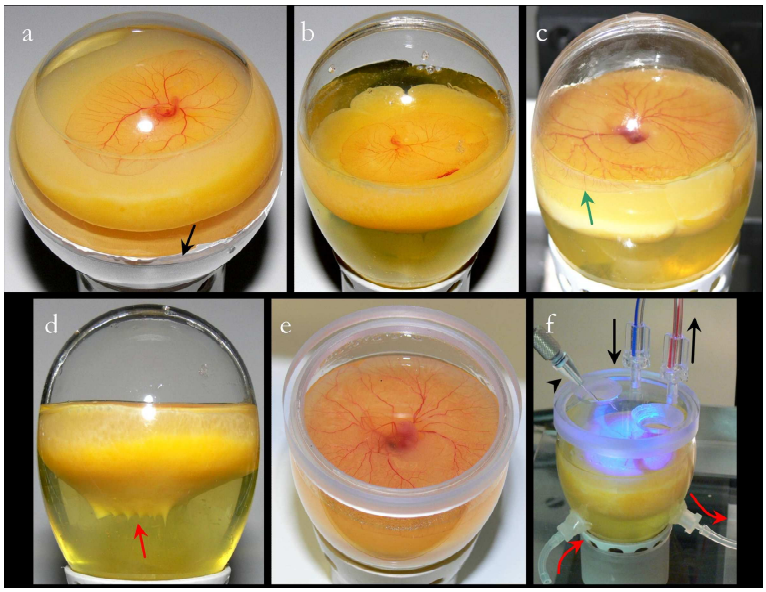

Figure 6.17: Chicken embryo in completely artificial and transparent egg system.

(a) Chicken embryo (96 hrs old) is separated from the air cavity with a porous Teflon membrane (arrow). (b), (c) and (d) Chicken embryo in egg shaped oval plastic container; (e) egg shaped oval plastic container with flat observation window at the top; transparent egg with installed fluidics (curved red arrow) for exchange of culture medium, channels for gaseous exchange with the possibility to use micromanipulation system for addition of cells/factors of removal of samples form to the system. (a), (b), (d) and (f) contain 36 hrs old, (c) and (e) contain 5 days old chicken embryo. In image (c) outer margin of the CAM touching the container wall is marked by green arrow. (d) Side view of the transparent egg showing the free border of the vitelline membrane (red arrow).

As described earlier, the system will not contain any chicken embryo in the long run, but cells will be cultured on the same principle. It is necessary to have such automated and integrated setup for future investigations. In spite of little success, there are a lot of questions remained unanswered that have to be addressed in future.

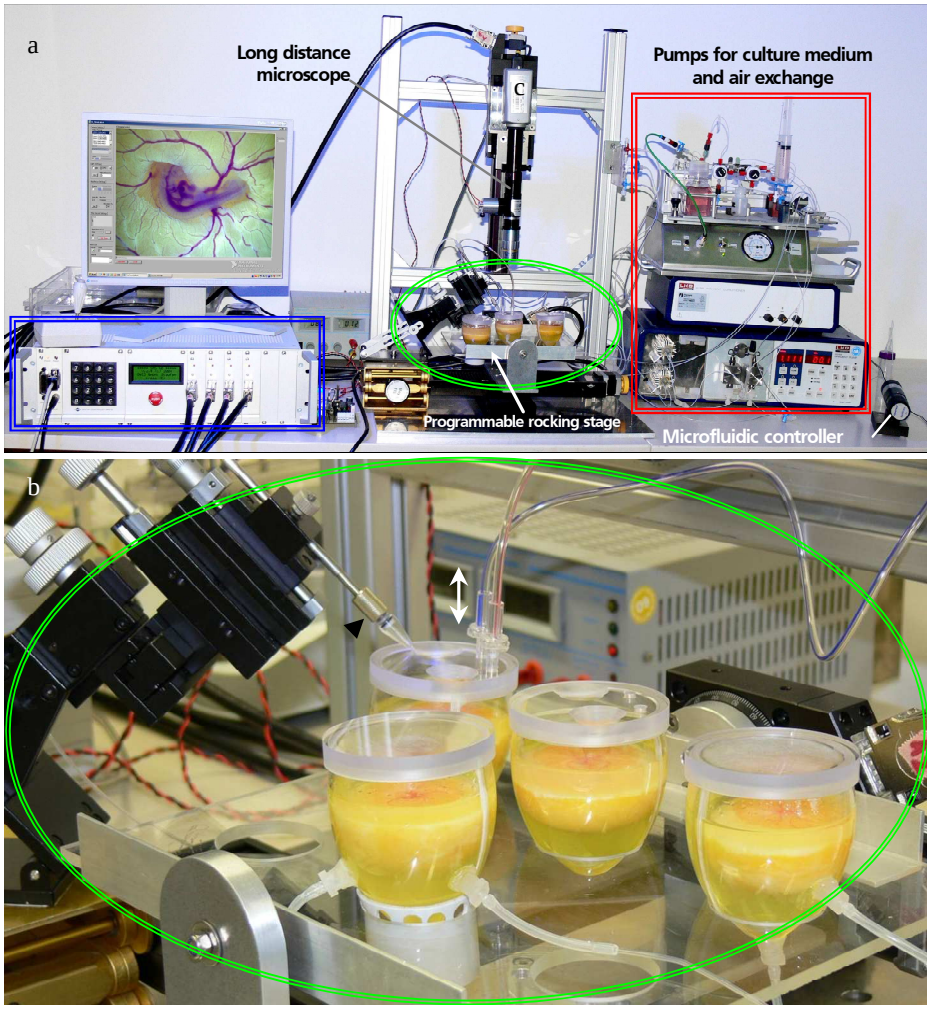

Figure 6.18: Complete technical setup for *in vitro* culture of cells at liquid | liquid interfaces.

The whole system consists of modified open system of avian culture, constructed long distance fluorescent microscope, constructed micromanipulator system (black arrowhead) with micro fluidic controller, a programmable rocking stage for automated rocking and sample changing (green ellipse in image a and image b), SMS 60 motor controller for controlling camera auto focus and automated rocking stage; red rectangle = automated pumping system (LKB 2249 gradient pump from Bromma, Sweden) with fluidic panel (evotec technologies, Germany) for medium exchange through fluidics, air channels for monitoring and exchange of air (double head white arrow), (c) camera.

6.8 Discussion

Even though Ca^{2+} is an essential macro-nutrient for the developing embryo and require in bulk for osteogenesis, the experiments with dried eggshell or eggshell without membrane revealed that the calcareous egg shell can induce inflammation of the CAM. Electron microscopy has revealed the fibrous and porous structure of the shell membrane, which prevents the egg contents contacting the calcareous shell. As described earlier, during embryonic development, around ID 10-12, the CAM becomes attached to the porous, acellular shell membrane adjacent to the eggshell but remains completely isolated. At the same time, the outermost ectodermal layer of the CAM

undergoes distinct differentiation into two major cell types, the capillary-covering and the villuscavity cells [57], and commences to transport calcium [337] with the concomitant expression of calcium-binding protein- CaBP [348] and carbonic anhydrase [351]. Calcium transport by the CAM is a development-specific function, beginning on ID 12-14, and continues until hatching [281, 337, 351]. In the process of calcium transport by the CAM, carbonic anhydrase is involved in the enzymatic acidification and dissolution of the shell mineral ($CaC0_3$) and/or the subsequent metabolic scavenging of the liberated $^{-}HC0_3$ [351]. For the windowing experiment of surrogate shell, it was important to prevent direct contact of CAM with calcareous eggshell. It is known that eggshell induce inflammation in the CAM [163]. For that reason, the egg shell was ground at least 1 mm beyond the shell membrane during preparation of the window in recipient egg. Dissection of the dead specimen also revealed the same story (image not shown), CAM attached firmly to the exposed egg shell at the window. Since the calcareous eggshell is the main source of Ca^{2+} for the developing embryo, and the artificial system contains no eggshell, the culture medium should contain Ca^{2+} in organic form (Ca lactate, $CaCl_2$ is rather harmful). It was not clear why some researchers used egg shell powder as calcium supplementation in complete artificial system [147].

There are some points to be considered for the construction of artificial systems that will replace the calcareous eggshell. Since such artificial systems will contain no eggshell, which is the main source of Ca^{2+} for the developing embryo, the alternative can be a sustained supply of Ca^{2+} to the system according to the necessities. As described earlier in chapter 4, Ca^{2+} can be supplied in organic form to the culture. Ca^{2+} is very important for the developing embryo and has to be supplied in bulk. Requirement is high especially during osteogenesis, addition of Ca^{2+} in such high concentration may be toxic to the embryo. Avian embryos gradually mobilise Ca^{2+} from the shell. This means that there should be a sustained supply of Ca^{2+} to the system. One opportunity could be to supply of Ca^{2+} through the installed fluidics dissolved in organic form in the culture medium (Figure 6.15, Figure 6.17). Another option could be to integrate Ca^{2+} with the new material used in exchange of the eggshell. This is a very important consideration in bone tissue engineering where the osteoblasts require a large amount of calcium for ossification of the bone. In traditional tissue engineering the limiting factor for the survival, proliferation, and differentiation of transplanted cells is

an insufficient supply of nutrients and oxygen. For mass transfer requirements, the angiogenesis of the tissue-engineered construct has to be considered too.

The materials mimicking the shell membrane has to be porous and biocompatible. Since the eggshell triggers inflammation in the CAM [163], incorporation of Ca^{2+} into the container material may lead to direct contact of CAM to the Ca^{2+} salts which might again cause inflammation. Since transparency is a desired material property in this aspect, gradual release of calcium from the artificial system may degrade the optical quality. It is necessary to undertake a thorough investigation to study the property of the shell membrane which will guide for the selection of the new material for construction of artificial egg.

The windowing experiment (Chapter 6.5) gave some clue about the properties of materials used to construct an artificial egg. Most of the window materials used in the windowing experiments are routinely used for cell culture in the laboratory and polyimide is the material of choice for fabrication of implantable micro electrode. The biocompatibility of the materials is beyond doubt. However, the results of windowing experiment with polyimide membrane came out with the worst outcome (Figure 6.12), maximum embryo survival was ID 11 and growth of the CAM blood vessels was not normal. On the other hand, polystyrene cover slips showed better outcome than other materials used for windowing of surrogate shell (Figure 6.10). Maximum embryo survival was day 19 of incubation and 60% embryos were viable at incubation day 18. However, the material is non-porous and no gaseous exchange is possible.

The window area covered only ≈3.6% of the CAM surface on 10^{th} day of incubation [32] which slightly blocks gas exchange and Ca^{2+} absorption from the surrogate shell; but the results of the experiments told a different story. They indicate that there is lot more than the biocompatibility if the growth of the CAM or the embryo is considered. Bioglass taken from FluoroDish™ is porous and predicted to be suitable for this experiment since gaseous exchange can take place across the bioglass window. But the result showed different scenario (Figure 6.9). The maximum survival of chicken embryo in surrogate shell with bioglass window was day 16. There was a sharp decrease in the viability of the chicken embryos at ID 9.

The window location at the surrogate shell is also an important factor that influence the embryo viability. In surrogate shell the CAM grows from the top and gradually grows towards the sharp end (since the opening is made at the blunt end to prepare surrogate shell and the shell is placed vertical during culture period). The shell is thicker at the poles than at the equatorial region and the rate of gas exchange is also not same. Cell culture Petri dish was placed at the pointed end of the egg as window and survival was longer (ID 20).

Question might arise regarding the necessity behind the construction of a completely artificial egg, as the avian egg is a complete system. It is advantageous to have complete artificial materials instead of natural eggshell because of the reproducibility of the system and known boundary conditions. It is necessary to understand all the boundary conditions associated with the culture system. Biological system is variable, sometimes unpredictable and non-reproducible. It is necessary to have a reproducible system constructed with materials of known property and understand the related boundary conditions where the results of the experiments can be predicted, than to have a system with stochastic behaviour. Such a system is necessary for understanding the mystery behind the defined cell migration, differentiation, tissue formation and organogenesis during embryogenesis. To reveal the secret will answer the question that the scientists had been looking for – what makes a complete organism from a single cell, that is not possible *in vitro*. This may lead to the way to culturing tissue or even organs in the lab from the stem cells.

However, a key point for tissue engineering is angiogenesis, which *in vitro* is not working in the constructed model systems untill now; this is also the main obstacle in current tissue engineering approach. Never the less, the technically modified open culture system is a very useful tool to study the complete process of embryogenesis especially angiogenesis. Since the modified open system has been modified to bring different imaging and assay method as close as possible to the embryo and was tasted against the gold standard of hatching of a viable bird, it will help to reveal the secret of angiogenesis *in ovo*, which is not possible in *in vitro* state.

6.9 Conclusion

Experiments in this chapter highlighted the basic difference between the avian egg as a culture system and the current *in vitro* culture - the changing boundary conditions. The egg shell is not only a housing for the growing cells and the future embryo, but also a Ca^{2+} reservoir and an intelligent ventilator that dynamically changes the property to regulate gas and water exchange to maintain the appropriate microenvironment for the differentiated cells. The shell membrane plays a vital role in regulation of gas exchange, water evaporation, Ca^{2+} regulation, provides support for CAM growth and much more. It is, therefore, necessary to study the physiochemical properties of the shell membrane of chicken eggs. In these experiments, it was not possible to replace the eggshell with traditional materials. Since such clever material is not available, it is therefore necessary to change incubation conditions to the needs of differentiated cells. Based on the experimental results in this chapter, some material properties can be highlighted which is useful for future design.

The results of the experiments showed that emphasis should be given on the following parameters while constructing an artificial transparent egg:

- The material should be increasingly porous to allow gradual increase of gaseous exchange to meet the changing demand of the growing embryo. This gradual increase of gaseous exchange can be achieved by medium exchange through the fluidics and adaptive changing of culture environment.
- Since materials with changing physical property is difficult to fabricate, an alternative should be a variable culture environment regulated in accordance with the changing microenvironment of the embryo.
- There should be a feedback control to regulate different physio-chemical status of the culture which can be easily done by implantation of biosensors and especially "**Lab-On-Chip-Technology**" can be implemented in conjunction.
- The material should be biocompatible. The best would be to study the structure of the shell membrane in detail and to find out the specific material property necessary.

- Should have the surface property similar to the shell membrane that allows the firm attachment and growth of CAM.
- Ca^{2+} in organic form can be supplied to the culture system by fluidics dissolved in the culture medium since constant supply of Ca^{2+} in high concentration may be toxic to the developing embryo.
- For better optical access, the material should be of high optical quality.

7 General discussion and outlook

From the technical and biotechnological point of view, the avian egg is a highly sophisticated, automated and dynamic culture system that changes boundary conditions permanently. Experimental investigations of avian eggs in this thesis reveal some striking features making them distinct as a culture system. Those include growth of cells at the liquid | liquid interface and the changing boundary conditions in accordance with the necessities. At this liquid | liquid interface follows the cell division, cellular migration, cell differentiation, and tissue formation during the process of embryonic development. During embryogenesis–dominated by cell microenvironment and orderly cell migration in groups – a single fertilized oocytes gives rise to a multicellular organism whose cells and tissues have adopted differentiated characteristics or fates to perform the specified functions of each organ of the body. Here the cells are not guided or dominated any solid substrate. During embryogenesis, (dominated by cell microenvironment) orderly cell migration in groups – form the germ layer, the tissue, organ and at last a functional organism. Apparently, this three-dimensional freedom of movement facilitates for cell division and migration as well as far reaching freedom of the embryo.

Table 7-1: Speciality of the avian egg as culture system

- Complete system in terms of the logistic requirement for the developing embryo
- Independent of mother other then temperature, humidity and mechanical movement
- The cell division stops as it cools after the egg is laied and resumes again on incubation
- The thick eggshell provides the structure, stability, camouflage and protection against bacterial invasion.
- Calcareous eggshell remains separated from the rest of egg contents and CAM by double layer of shell membrane since bare eggshell cause inflammation.
- Gradual and permanent change of boundary conditions according to the changing microenvironment
  - Maintains CO_2 rich environment necessary for early embryogenesis
  - Gradual thinning of the egg shell with gradual mobilization of Ca^{2+} from the egg shell
  - Gradual increase of O_2 and CO_2 permeability
  - Gradual increase of water loss
  - Thinning of the eggshell helps for external pipping of the embryo.
- Automated resorption of Ca^{2+} from the egg shell by CAM, which is completely separated by the shell membrane, and synthesize all necessary factors for Ca^{2+} resorption.
- Complete automated process with feedback control to maintain the microenvironment for the dividing and differentiating cells
- Active supply with rich capillary network of CAM
- The embryo consumes the remains of the egg contents and comes out of the egg leaving nothing behind except for the extraembryonic membranes.
- Full sterility in a contaminated environment

This thesis was the first and a very important step towards the realization of a new culture system for *in vitro* cell culture at the liquid | liquid interface imitating the same natural principle of an avian egg. The open culture system for the avian embryo with transparent window and culture of avian embryo till hatching indicate the progress towards the construction of complete artificial system and is the proof for the feasibility of such system. Even though it was not possible to demonstrate lineage specific differentiation of stem cells *in vitro* or to culture tissue *in vitro* out of them. But this phenomena is present in avian egg which attained the perfection through the evolution of million of years and gradual modification of the open system will enable that in future. Since the avian egg is complete and independent of the mother, it is easier to study the cellular processes in the developing embryo. The constructed open system in conjunction with the micro-imaging and micromanipulation system brought the field an important step ahead.

In spite of considerable success of current tissue engineering approach, it has reached to a dead end- tissue engineering is not possible without angiogenesis and it is also not possible to initiate angiogenesis in the tissue engineering constructs. The limiting factor for the survival, proliferation, and differentiation of transplanted cells is the sufficient supply of nutrients and oxygen. The current tissue engineering approach relies entirely on diffusion processes for this supply. Furthermore, to supply tissue-engineered constructs thicker than a few millimetres, initial vascularisation from the surrounding host tissue is necessary.

The CAM has become an ideal substrate to investigate the process of embryonic angiogenesis and vasculogenesis at the cellular level. However, to study these phenomena, an open system is required that allows access of different high-resolution imaging methods. µMRI provides high-resolution and non-invasive imaging possibility, which is very advantageous for *in ovo* application but seems not to be a solution with its disadvantages, especially the motion artefacts and longer image acquisition time. The modified open system on the contrary allows different optical imaging methods and provides a very high resolution, real time *in ovo* imaging at the cellular level. Co-application of micromanipulation system with the constructed microimaging system will allow contrast labelled cells/samples to implant/inject into the embryo and image to

follow the fate; withdrawal of sample from the desired location will allow parallel biochemical and histological analysis of the samples to extract additional informations.

Table 7-2: Summerised results of the complete experiments in this Ph.D. work

Methods/Procedure	Results/Comment
Noninvasive 3D Imaging of avian embryogenesis *in ovo* with µMRI	Allowed very high resolution (39 µm ×39 µm), 3-D imaging, but limited by too long image acquisition time, motion artefacts, and smaller probe size. µMRI was not a solution for the current study.
Avian embryo culture in open system • Bantam embryo in broiler egg shell –67 % hatching • Broiler embryo in broiler shell –38 % hatching • Quail embryo in bantam egg shell – 5% hatching • Quail embryo in complete artificial system- 0%hatching	For the first time, different influential parameters for the open culture system were identified. The open system is a very useful tool for further experiments that will enable to construct a liquid|liquid interface culture system for *in vitro* cell culture.
Technical modification of the open system for optical imaging *in vivo* • Construction of especial lid for the open system for optical imaging. • Construction of long distance fluorescence microimaging and micromanipulator system, • Construction of especial flexible microelectrode array for *in vivo* application	Imaging of the complete chicken development from the beginning of incubation until hatching with changing magnification to image the desired part of the developing embryo. Constructed long distance fluorescence microimaging and micromanipulator system enabled to inject /remove fluroscence labelled cells into the transperant embryo at the early stage that will help to study cellular physiology *in ovo*.
Technical modification of the open system towards liquid|liquid interface culture • Scanning electron microscopy of egg shell and SM to study the ultrastructure Open system consisted with • Surrogate shell without shell membrane- 0% hatching • Dried surrogate shell –0% hatching • Windowed of surrogate shell with ♦ Polystyrene- max. survival ID 20 ♦ Bioglass - max. survival ID 17 ♦ Polyamide - max. survival ID 14 ♦ Cell culture Petridish - max. survival ID 20 ♦ Cling film - max. survival ID 16 ♦ Plexiglas - max. survival ID 15 ♦ Complete artificial and transparent egg - maximum survival was ID 5	The experiments provided an outlook for construction of a complete artificial liquid|liquid interface *in vitro* cell culture system for routine cell culture as well as for Tissue Engineering. Eventhough it was not possible to culture embryos untill hatching (which is regarded as the assessment parameter for the system) and angiogenesis remains to be a key point to be solved for the tissue engineering in the complete artificial and transparent egg. Never the less, the process of angiogenesis works perfect in the avian egg open culture system. Careful study and modification of this system will enable to construct a complete artificial system adopting the same physiochemical principle of avian egg where the problem of angiogenesis will be overcome in future.

One distinguishing feature of avian egg as a culture system is the permanent changing boundary conditions which adapt in accordance with the changing demand of the dividing and differentiating cells. Unlike the static boundary conditions in *in vitro* culture of cells, egg shell with its membrane provides more dynamic conditions to the cells inside. By the time the egg is laied, the embryo reaches the blastoderm stage with several thousand non-differentiated cells. Cell division stops due to drop of temperature. With incubation, cell division resumes. O_2 is toxic to the cells at this stage and therefore the climate is rich in CO_2 to support the cellular demand. Porous egg shell permits limited gas exchange at this stage to maintain gaseous status. With further growth, the

O_2 demand of the embryo increases. This increasing demand is fulfilled by the gradual increase in gas exchange across the shell with gradual thinning of the egg shell with the mobilization of Ca^{2+} by CAM.

The egg shell is not only a housing for the dividing cells inside, but also a cleverly designed reservoir of Ca^{2+}. It is toxic to the embryo in inorganic form but $CaCO_3$ gives mechanical stability of the shell. That is why the egg shell is completely isolated from the egg contents by a double layered shell membrane. As the embryo grows, it requires Ca^{2+} in bulk for osteogenesis. CAM gradually mobilizes Ca^{2+} from the shell.

In this thesis was concerned regarding the preliminary study of the feasibility to construct a liquid│liquid interface culture system to culture cells at the interface of two immiscible liquids. This method is based on the same natural principle as the avian embryo grows inside the egg. From a technical and biotechnological perspective, the chicken can be seen as an interface of two immiscible liquids, where the blastoderm develops at the interface between a protein rich in water (egg white / albumen) and lipid (egg yolk). As in nature, cells can also be cultured at the liquid│liquid interface in the similar way. Two immiscible liquids placed together separate from each other creating an interface. One of these liquids can be tissue culture medium and other liquid, relatively immiscible with the first one, is hydrophobic, higher density than water and non-toxic to living calls. In this cultivation method, anchorage dependent animal cells can anchor, spread and grow at the interface [151-153]. Different artificial and natural membranes, nano/microstructures, biometrics and self assembling nanofibres can be placed at the interface for culturing anchorage dependent cells.

The cultivation of anchorage-dependent animal cells is performed at the interface between solid substrate and liquid culture medium, where the whole process is governed by the physiochemical properties of the solid substrate. Cellular phenotype often dominated and limited by the cellular microenvironment and technical boundary conditions of the substrate. Inappropriate alteration to cellular microenvironment and rigid substrates hinder cellular communication, cell division, differentiation and migration. This results into a flat layer of homogenous cell sheet, which is unlike tissue consisting of inhomogeneous cells performing a specific function in coordination. The current technology of *in vitro* culture in flasks or on dishes developed from Petri dishes and nutrient-gel-surface culture of microbiology. It is less automated, allows insufficiently defined microenvironment of cells and limited and dominated by technical

boundary conditions of the flask. In general, these methods are used to keep and culture cells outside the organism.

In 1964 Rosenberg introduced the use of a fluid substrate for the growth of both transformed and anchorage-dependent cells [284, 285]. In this method a cell suspension is introduced over an inert hydrophobic liquid having a density greater than that of the aqueous medium, and cells are observed to spread and divide at the liquid|liquid interface between the two immiscible phases. As is the case for solid substrates, the cells do not interact directly with the interface but rather with proteins that adsorb to the interfacial junction. One of these liquids can be tissue culture medium and other liquid, relatively immiscible with the first one, is hydrophobic, higher density than water and non-toxic to living calls. In this cultivation method, anchorage dependent animal cells can anchor, spread and grow at the interface [151-153]. As is the case of solid substrate, the cells do not directly interact with the interface, but rather the proteins adsorb to the interfacial junction. Different membranes can be place at the interfacial zone, which may include natural and synthetic membranes, micro and nano structures and biomatrices.

This method of cell culture has the advantage of being exceptionally homogeneous and reproducible when compared with hydrophobic solid surfaces, which, in general, have polar molecular inhomogeneities. In addition, cells grown on such substrates can be transferred by simply pipetting the cell layer. Such procedure of cell harvesting is especially important where the effect of trypsin or other proteolytic enzymes or chelating agents to passage cultured cells can be avoided. It is here to be mentioned that the compositions of cell membrane proteins are changed by enzymatic treatment or by mechanical scraping [301]. Such a procedure is of particular interest in that it obviates the use of trypsin or other proteolytic enzymes or chelating agents to passage cultured cells and avoids the uncertain effects of such treatments. If the adsorbed serum proteins at the interface are crosslinked by using glutaraldehyde or if a bimolecular layer of proteins is formed by using polylysine as a base coat [88], the patterns of cell growth can be altered significantly. This simply means that the cell growth can be regulated as desired. In addition this method is free from the influence of the technical boundary conditions of the substrate and allows freedom to the cellular migration similarly in the developing avian embryo at the interface of egg white and egg yolk.

It is a matter of regret that since the original work was published in 1964, there seems to have been relatively little interest in the growth of cells on fluid substrates in spite of many advantages not afforded by solid substrates in culture. There had been a patent regarding cell culture on a large number of small protein-coated droplets of a first liquid dispersed in a second liquid in the nature of an emulsion [102]. One of these liquids is to be a sterile aqueous tissue culture medium and the other liquid, relatively immiscible with the tissue culture medium, is non-toxic to living cells. Hanging drop preparation is perhaps one such example where cells are cultured at the liquid | gas interface. Nevertheless, this process is different from the natural process of liquid | liquid interface culture proposed in this thesis.

The goal of this thesis was to gradually adapt the avian egg for interface culture system to culture mammalian cells. Keeping the final goal in mind, first the process of avian embryogenesis was studied non-invasively using µMRI with highest possible resolution. In addition, the potential of tracking the SPION labelled cells injected into the fertilised egg was also considered. With its drawbacks, µMRI was not feasible for *in ovo* application for realtime imaging. Next, the avian embryo was cultured in the open system and was brought to hatch. Later the open system was modified for optical imaging to allow the study of the vital process of embryonic development at the liquid | liquid interface inside avian egg with optical imaging methods. This opened a new door for studying the cellular migration, cell differentiation, organogenesis and, eventually, the embryogenesis.

Scientists are now starting to realize the disadvantages of *in vitro* cell culture methods but are still moving in the same non-physiological way; trying to mimic the three dimensional environment *in vitro*: to grow cells in 3-dimentional culture. However, there is no emphasis on liquid | liquid interface culture. Cellular microenvironment can be very well studied by exploiting the independency and the completeness of the avian egg. It was not possible to realise the new culture system in the short time span of this thesis, but further investigations in future will allow the construction a liquid | liquid interface culture system for *in vitro* culture of cells, which will help to remove the drawbacks of the traditional *in vitro* cell culture system.

8 Bibliography

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Publications related to this thesis

Peer reviewed journals

1. Haque, S., Fuhr, G., "New system for real time study of *in vivo* migration and differentiation of stem cells". Microsystem Technologies (submitted)
2. Haque, S., Gorjup, E., Fuhr, G., "Effect of surrogate shell on hatching of avian embryo in explantation culture". Manuscript.

Symposium

Fuhr, G. R., Gorjup, E., Haque, S., "*In vitro*-culture of animal cells – a biological and technical challenge". 3rd IBEC Symposium on Bioengineering and Nanomedicine in Barcelona (Spain), 01.05 – 02.05.2010

Patents

1. Kruse, C., Fuhr, G. R., *Verfahren und Vorrichtung zum Kultivieren von Stammzellen*, Deutsches Patent- und Markenamt, 102004025086 (2007).
2. Fuhr, G. R., von Briesen, H., Gorjup, E., Kruse, C., *Verfahren und Kultivierungseinrichtung zur Kultivierung biologischer Zellen*, Deutsches Patent- und Markenamt, 102006006269 (2007).

Annexes

Cling-film 'glue': Cling-film glue was made by adding 0.1 ml Amphotericin (500µg/ml) and 0.2 ml penicillin/streptomycin mix (10,000 IU/ml Pen and 10,000 IU/ml streptomycin) to 10 ml freshly collected thin albumen

Protocol for egg sterilisation: The wash solution consisted of 5% aqueous solution of Hypo chloride (200-500 ppm) (Sodium hypochlorite solution from Sigma-Aldrich GmbH). Eggs are washed in a mixture of 10l H_2O + 70 ml Sodium hypochlorite solution at 37 °C for 3 minutes. After washing, eggs are dried at room temperature and stored in a refrigerator at 14 °C.

Prussian blue staining:

Solutions:

A. Potassium Ferro cyanide Solution (filter)

Potassium Ferro cyanide - 1 g

Distilled Water - 50 ml

2% Hydrochloric Acid (1ml in 50ml) - 50 ml

B. Neutral red

Procedure: First, cells were fixed in 10% Para formaldehyde solution for 10 min, washed in PBS, and stained with a freshly prepared solution of 5% potassium ferrocyanide and 10% hydrochloric acid for 20 minutes. Than stained with neutral red for 1-5 minutes and rinsed with water and dehydrate in 96% alcohol two times then 100% alcohol. After that cleared in xylene (Xylol). Photographs were taken of representative fields.

Pen Strep (Penicillin/Streptomycin) solution: contains 10,000 units/ml Penicillin and 10,000 µg/ml Streptomycin used without dilution.

Amphotericin B: Contains 250 µg/ml Amphotericin B used without dilution.

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2006-2010	Ph.D. thesis at the Fraunhofer-Institute for Biomedical Engineering (IBMT) in St. Ingbert, Germany

Professional experiences

1999 – 2000	Internship as a General Physician and Surgeon in Chittagong Medical College Hospital, Chittagong, Bangladesh.
2002- 2003	Lecturer, Department of Physilogy, Medical Faculty, Gono University, Savar, Bangladesh
2003	Medical Officer, shibganj Thana Health & Family Welfare Complex, Chapainowabganj, Bangladesh
2005 - 2006	Auxiliary scientist in Neuromonitoring group in Fraunhofer IBMT at the Fraunhofer-Institute for Biomedical Engineering (IBMT) in St. Ingbert, Germany